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Circulating microRNAs and Renal Cell Carcinoma

Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Informação Técnica

TÍTULO:
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Em memória do meu avô Manuel…

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less” - Marie Curie

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Ao terminar esta etapa do meu percurso académico, gostaria de agradecer a todos aqueles que, direta ou indiretamente, contribuíram para a realização deste projeto.

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Esta é para ti!

Abbreviations

A

ACVR1B	activin A receptor, type IB
AGO	Argonaute
AKT	protein kinase B

C

CASP8AP2	Caspase Associated Protein 2
ccRCC	clear cell Renal Cell Carcinoma
cDNA	complementary deoxyribonucleic acid
COX10	Cytochrome C Oxydase 10

D

DNA	Deoxyribonucleic acid

E

E2F3	E2F Transcription Factor 3
EAU	European Association of Urology
EDTA	Ethylenediamine Tetraacetic Acid
EFNA3	Ephrin-A3
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMVs	Extracellular microvesicles
EPO	Erythropoietin
ERK	Extracellular-signal-regulated kinases

F

FBS	Fetal Bovine serum
FGFLR1	Fibroblast Growth Factor 1

G

GLUT1	Glucose Transporter 1

H

HIF-α	Hypoxia Induclible Factor alpha
HOXA1	Homeobox A1
HRAS	Harvey rat sarcoma viral oncogene homolog

I

IARC	International Agency for Research on Cancer
ISCU1	Iron-sulfur Cluster Assembly Enzyme 1
ISCU2	Iron-sulfur Cluster Assembly Enzyme 2
ITS	Insuline-transferrine-selenium

M

MAPK	Mitogen-activated protein kinases
MET	MET proto-oncogene, receptor tyrosine kinase
MEK	MAP kinse-ERK kinase
miRNAs	microRNAS
miRs	microRNAS
mRCC	metastatic Renal Cell Carcinoma
mRNA	messenger ribonucleic acid

P

PDGF-β	Platelet Derived Growth Factor Beta
PDK	Phosphoinositide-dependent kinase
pH	potential of Hydrogen
PI3K	Phosphatidylinositide 3-kinases
PTEN	Phosphatase and Tensin homolog
PTP1B	Protein Tyrosine Phosphatase, non-receptor type 1

R

RASSF7	Ras association (RalGDS/AF-6) domain family (N-terminal) member 7
RCC	Renal Cell Carcinoma
RISC	RNA induced silencig complex
RNA	Ribonucleic acid

S

SDHD	Succinate Dehydrogenase Complex Subunit D

T

Tcf7l2	Transcription Factor 7-like 2
TGF-α	Transforming Growth Factor alpha

V

VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	von Hippel-Lindau

W

WHO	World Health Organization

X

XPTO5 Exportin 5

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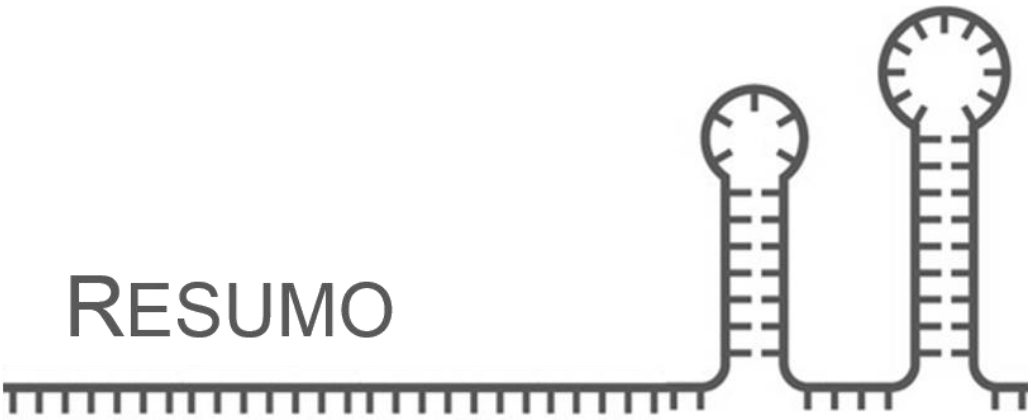
RESUMO



Resumo

O carcinoma de células renais (CCR) é a neoplasia renal mais frequentemente diagnosticada no adulto (cerca de 90% dos casos) e é também considerada a neoplasia urológica mais letal. Esta neoplasia é responsável por mais de 100 000 mortes por ano a nível mundial, observando-se também um aumento das taxas de incidência e mortalidade (2-3% por década). A inexistência de um teste de *screening* para a deteção precoce e *follow-up* contribuí para o facto de 1/3 dos doentes serem diagnosticados com doença metastática e cerca de 20-40% do doentes submetidos a cirurgia desenvolverem metástases. A hipótese da existência de uma comunicação celular dependente de exossomas tem vindo a revolucionar a biologia molecular, criando espaço para a reformulação da hipótese que suporta o envolvimento do microambiente tumoral como indutor/modulador do processo metastático. Dos vários tipos de ácidos nucleicos capazes de serem transportados pelos exossomas, são de destacar os microRNAs (miRNAs). Os miRNAs são pequenas moléculas de RNA não codificante responsáveis pela regulação pós-transcricional de inúmeros genes e têm sido amplamente estudados em Oncologia uma vez que estão desregulados no cancro, podendo influenciar o desenvolvimento neoplásico.

O objetivo do presente estudo foi o estabelecimento de um perfil de expressão de miRNAs em circulação que possa ser utilizado como biomarcador de diagnóstico e/ou prognóstico em doentes com CCR. Para tal foi realizado um estudo *in vitro* em linhas celulares de rim, uma normal e uma metastática (HK-C8 e FG-2, respetivamente), a fim de estabelecer um perfil de miRNAs desregulados em CCR. Numa segunda fase, o perfil de miRNAs obtido foi validado em amostras de plasma de doentes com CCR (n = 50) e em indivíduos saudáveis (n = 50), assim como foi analisado o seu impacto na sobrevivência global dos doentes com CCR.

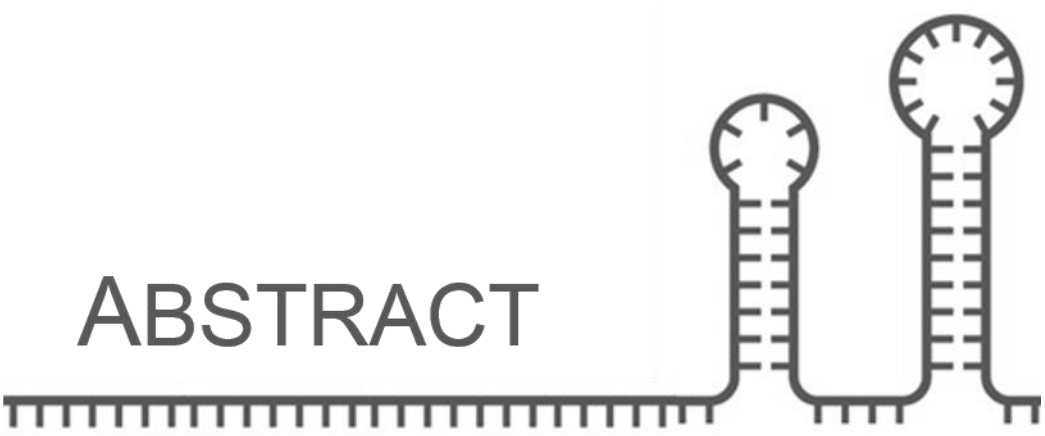
Dos 5 miRNAs estudados nas linhas celulares, apenas o miR-210, o miR-218 e o miR-1233 apresentaram níveis de excreção mais elevados na linha FG-2 (miR-210: *fold-increase* = 2.64, P=0.020; miR-218: *fold-increase* = 302, P=0.002; miR-1233: *fold-increase* = 10.63, P=0.021). Quando analisámos os níveis de expressão destes miRNAs na população, observamos que o miR-210, miR-218 e miR-1233 apresentavam níveis de expressão aumentados nas amostras de plasma dos doentes com CCR comparativamente aos indivíduos saudáveis (miR-210: *fold-increase* = 5.24, $P \leq 0.001$; miR-218: *fold-increase* = 27.10, $P \leq 0.001$; miR-1233: *fold-increase* = 52.34, $P \leq 0.001$). Observámos também que o aumento dos níveis de expressão destes miRNAs se

encontrava associados a características clinicopatológicas, nomeadamente: tamanho tumoral superior (miR-210: *fold-increase* = 7.84, $P \leq 0.001$; miR-1233: *fold-increase* = 8.05, $P = 0.007$), maior grau de Fuhrman (miR-1233: *fold-increase* = 5.13, $P = 0.011$), presença microinvasão vascular (miR-210: 3.97 *fold-increase*, $P = 0.049$; miR-1233: *fold-increase* = 8.40, $P = 0.012$) e presença de metástases no momento do diagnóstico (miR-210: *fold-increase* = 4.63, $P = 0.005$; miR-218: *fold-increase* = 16.68, $P = 0.014$; miR-1233: *fold-increase* = 5.43, $P = 0.032$). Adicionalmente, observámos também que, doentes que expressam níveis elevados de miR-210 e miR-1233 em circulação, apresentam uma sobrevivência global menor (Log Rank test, $P = 0.036$).

A hipóxia é um evento primário no CCR, devido à alteração do gene *VHL*, e é responsável pela indução da angiogénese e vascularização necessárias ao desenvolvimento tumoral. Tanto o miR-210 como o miR-1233 são induzidos pela hipóxia e parecem atuar sinérgicamente para manter a resposta ao estado de hipóxia por parte da célula, uma vez que levam à sobreexpressão de genes relacionados com a proliferação, angiogénese, metastização e inibição da apoptose, o que se traduz num pior prognóstico e numa menor sobrevivência global.

Deste modo, e com base nos resultados obtidos, podemos concluir que o miR-210 e o miR-1233 são bons candidatos a biomarcadores de prognóstico e agressividade em CCR.

ABSTRACT



Abstract

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney, accounting for approximately 90% of kidney neoplasms and 3% of all adult malignancies. Worldwide mortality as a result of RCC currently exceeds 100.000 patients each year, with the incidence and mortality rate increasing by 2-3% per decade. This reality and the nonexistence of a standard screening test, contributes to the fact that one-third of patients are diagnosed with local invasive disease or metastatic disease. Moreover 20-40% of RCC patient's submitted to surgical nephrectomy will also develop metastasis. The hypothesis of exossome-dependent signaling between cells has been revolutionizing molecular biology and giving space for reformulating the hypothesis of tumor microenvironment as modulator of the metastatic process. Among the cargo that can be transported through exosomes we can find microRNAs (miRNAs). miRNAs are small non-coding RNAs that are responsible for the regulation of numerous genes at a post-transcriptional level. MiRNAs have been widely studied in oncology since they have been proved to be deregulated in cancer and also have influence in cancer development.

The aim of this study was the establishment of a circulating miRNA expression profile that could be used as a biomarker of diagnosis and/or prognosis in RCC patients. In order to do that we first performed an *in vitro* study using renal cell lines, one normal and the other metastatic (HK-C8 and FG-2, respectively), to establish a profile of deregulated miRNAs in RCC. In a second phase, we validated the miRNA profile obtained in plasma samples of RCC patients (n = 50) and healthy individuals (n = 50), and also evaluated its impact in the patients overall survival.

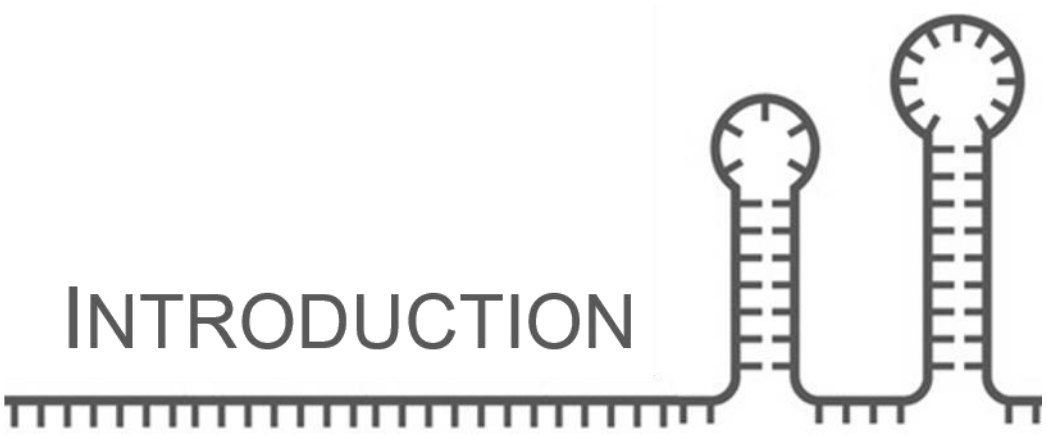
From the 5 miRNAs studied in the cell lines, only miR-210, miR-218 and miR-1233 presented higher circulating expression levels in the FG-2 cell line (miR-210: fold-increase = 2.64, P=0.020; miR-218: fold-increase = 302, P=0.002; miR-1233: fold-increase = 10.63, P=0.021). When we analyzed the expression levels of these miRNAs in the population, we observed that miR-210, miR-218 and miR-1233 presented higher expression levels in plasma samples of RCC patients compared to healthy individuals (miR-210: fold-increase = 5.24, $P \leq 0.001$; miR-218: fold-increase = 27.10, $P \leq 0.001$; miR-1233: fold-increase = 52.34, $P \leq 0.001$). We also observed that the increase of the expression levels of these miRNAs were associated with clinicopathological characteristics, namely: higher tumor size (miR-210: fold-increase = 7.84, $P \leq 0.001$; miR-1233: fold-increase = 8.05, P=0.007), higher Fuhrman nuclear grade (miR-1233: fold-increase = 5.13, P=0.011), microvascular invasion (miR-210: 3.97 fold-increase, P=0.049; miR-1233: fold-increase = 8.40, P=0.012)

and presence of metastasis at the time of diagnosis (miR-210: fold-increase = 4.63, *P*=0.005; miR-218: fold-increase = 16.68, *P*=0.014; miR-1233: fold-increase = 5.43, *P*=0.032). Additionally, we observed that patients that had higher expression levels of both miR-210 and miR-1233 presented a lower overall survival (Log Rank test, *P* = 0.036).

Hypoxia is a primary event in RCC, due to the loss or mutation of *VHL* gene, and is a well-established inducer of angiogenesis and vascularization, necessary for further tumor development. Both miR-210 and miR-1233 are induced by hypoxia and they appear to act synergically in order to maintain the hypoxic response from the cell since they lead to the overexpression of genes related to proliferation, angiogenesis, metastization and inhibition of apoptosis, which culminates in a poor prognosis and a lower overall survival.

Thus, based on these results, we conclude that miR-210 and miR-1233 are good candidates for biomarkers of prognosis and aggressiveness in RCC.

INTRODUCTION



1. Introduction

1.1. Cancer: general concepts

Cancer has revealed itself as an emergent public health problem worldwide. Nowadays it represents the first and the third cause of death in the developed countries and in the less developed countries, respectively [1]. According to GLOBOCAN 2012, the International Agency for Research on Cancer (IARC) online database, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, registered in 2008 [2]. Estimates prevalence rates for 2012 show that there were 32.6 million people (over the age of 15 years) alive who had had a cancer diagnosed in the previous five years [3]. Projection based on the GLOBOCAN 2012 estimates predicts a substantive increase to 19.3 million new cases per year by 2025, due to growth and ageing of the global population [2].

Despite the achievements in the field of Oncology, whether in the early detection of some neoplasias or in the development of new treatments more effective in disease management, the upcoming of resistance to these treatments has become a difficult obstacle to overcome. Cancer is a heterogeneous disease, with different etiology and natural history that develops trough the interactions between environmental and genetic factors, involving deregulation of multiple pathways responsible for the fundamental cell processes, such as death, proliferation, differentiation and cell migration [4].

In the beginning of the process of carcinogenesis the cell must undergo a change, genetic and / or epigenetic, which may lead to changes in gene activity through chromosomal rearrangements and translocations, deletions, insertions, amplification of certain genes and point mutations, giving the cell the potential to become a neoplastic [5]. Subsequently, multiple events occurs that result in the disruption of key cellular processes such as cell proliferation and apoptosis, which in turn lead to the growth and development of malignancies. The mutations acquired during neoplastic development make the cells less sensitive to apoptotic signals, cell growth stop and destruction by the immune system. Cumulatively, the self-sufficiency of these cells in growth factors provides them with greater proliferative capacity, angiogenesis and invasive potential. These factors combined with increase in inflammation, genomic instability and cell energy deregulation, favor cancer development and systemic dissemination, enhancing the metastatic process [6, 7].

However, despite the existing knowledge of several genetic factors on tumor pathophysiology, understanding the complex molecular mechanisms underlying its

development remains incomplete, implying that besides the intrinsic malignant properties of tumor epithelial cells, other factors such as microenvironmental changes may modulate progression, invasion and metastasis [8].

Over the past decade, tumors have increasingly been recognized as organs whose complexity approaches and may even exceed that of normal healthy tissues. When analyzed from this point of view, the biology of a tumor can only be understood by studying the individual specialized cell types within it as well as the "tumor microenvironment" that they construct during the course of tumorigenisis. This approach contrasts with the earlier view of a tumor as nothing more than a cluster of transformed cells standing alone, whose entire biology could be understood by elucidating their cell-autonomous properties.

Cancer cells in primary tumors are surrounded by a complex microenvironment. This microenvironment is composed by numerous types of cells including endothelial cells of the blood and lymphatic circulation, stromal fibroblasts and a variety of bone-marrow-derived cells including macrophages, myeloid-derived suppressor cells, TIE-2 expressing monocytes and mesenchymal stem cells (Figure 1) [9].

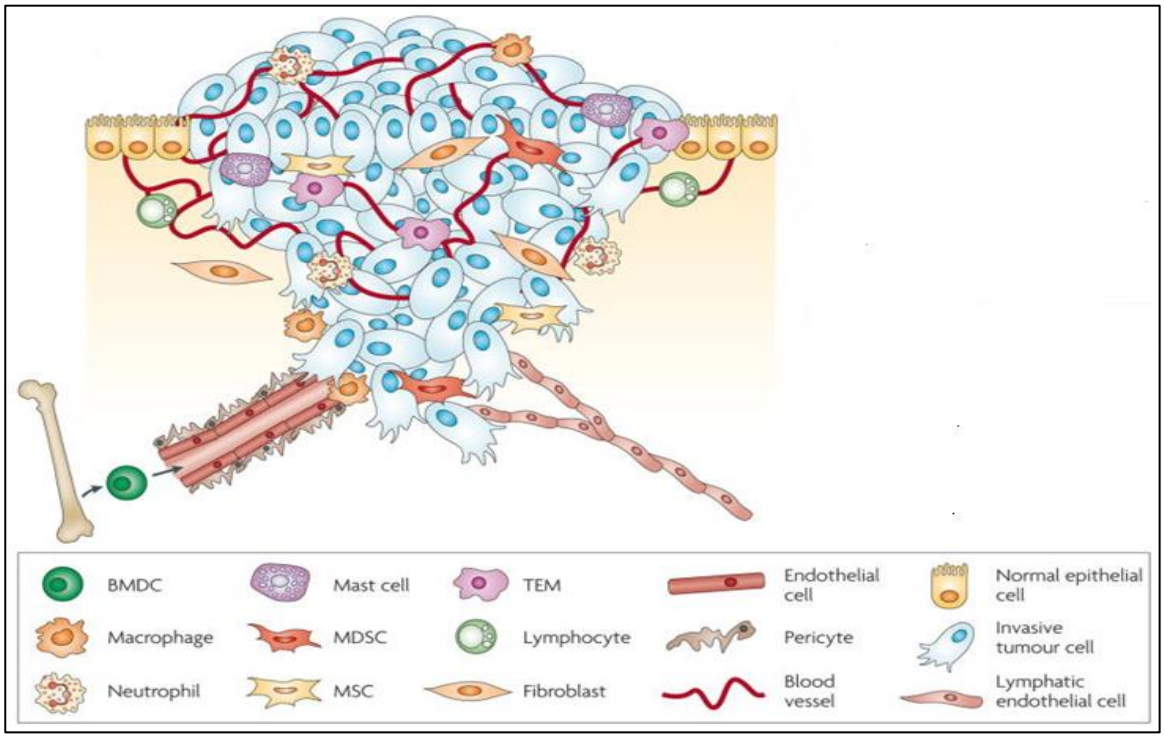


Figure 1 - The tumor microenvironment. (Adapted from Joyce *et al.* 2009 [9]).

Environmental alterations, together with natural viability in hereditary traits, continuously shape species. The tumorigenic process shares many similarities with the evolution of ecosystems; within tumors, a continuous selection exists for tumor cells with the highest survival and proliferative advantage. Moreover, tumors persistently shape their microenvironment, thereby establishing an abnormal ecosystem surrounding them [10]. Mathematical modeling studies, combined with experimental data, suggests that microenvironmental conditions, such as hypoxia and heterogeneous extracellular matrix, provide selective pressure for tumor evolution and progression to invasion phenotypes [11, 12].

One way of microenvironment shaping is through paracrine and/or systemic signaling between cells. This type of intercellular communication is made through exchange of biomolecules between cells and it's essential for functional integrity of multicellular organisms. Shed extracellular membrane vesicles (EMVs) serve to shuttle bioactive molecules between cells, and their cargo can modulate the extracellular environment [13]. The best characterized EMVs are the exossomes, 50- to 100-nm vesicles generated intracellularly in multivesicular bodies (MVBs) and released upon MVBs with the plasma membrane [13, 14].

Exossome-mediated cell communication includes, but is not restricted to, direct activation of cell-surface receptors on recipient cells, transfer and translation of mRNAs, transfer of microRNAS (miRNAs) and silencing of mRNA targets, transfer of functional proteins and the induction of cell signaling pathways upon their internalization and also genomic DNA and cDNA [13, 15]. Thus, exosomes can select bioactive molecules and propagate the horizontal transfer of their cargo and, consequently, have an enormous impact on tumor growth, survival and spread, along with demonstrated effects on many stages of tumor progression, angiogenesis, escape from immune surveillance, extracellular matrix degradation and metastasis [14, 16, 17]. Since they are released into the circulation, exosome-dependent signaling may occur not only locally, but also in a paracrine and systemic manner, which can have a direct impact in tumor progression and in metastization process (Figure 2) [15].

Figure 2 - Schematic model of exossome secretion in cancer cells. Exossome membranes are enriched in cholesterol, sphingomyelin, and ceramide, as well as lipid raft associated proteins. These components allow exosomes to be highly stable in numerous body fluids. Exosomes released from cancer cells can be taken up by neighboring cells and are capable of inducing pathways involved in cancer initiation and progression [14].

Given that this area is still underexplored, elucidating the nature of tumor cell interactions and the mechanisms of co-evolution of tumor cells and their microenvironment it's crucial for our understanding of what drives tumor initiation and progression and for the development of better cancer preventative and therapeutic interventions.

1.2. Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney, accounting for approximately 90% of kidney neoplasms and 3% of all adult malignancies [18, 19]. According to the European Association of Urology (EAU), there is a 1.5:1 predominance of new cases diagnosed in men over women, with a peak incidence occurring between the age of 60 and 70 years [19]. Worldwide mortality as a result of RCC currently exceeds 100.000 patients each year, with the incidence and mortality rate increasing by 2-3% per decade [20].

Incidence and mortality rates of RCC also show a geographic variation: the highest

incidence rates are observed in Northern America, Western Europe and Australia, whereas the lowest are observed in India, China and Africa. The highest mortality rates are registered in the European continent, mainly in the Central and Eastern countries, followed by the western and southern regions (Figure 3) [21]. In 2008, the number of new cases diagnosed in the European Union was 88 400 and the number of kidney cancer-related deaths was 39 300 [22].The geographic disparity observed in RCC incidence rates worldwide can be attributed to differences in diagnosis frequency, access to health care, genetic inheritance and prevalence of certain lifestyle habits and/or environmental risk factors [23].

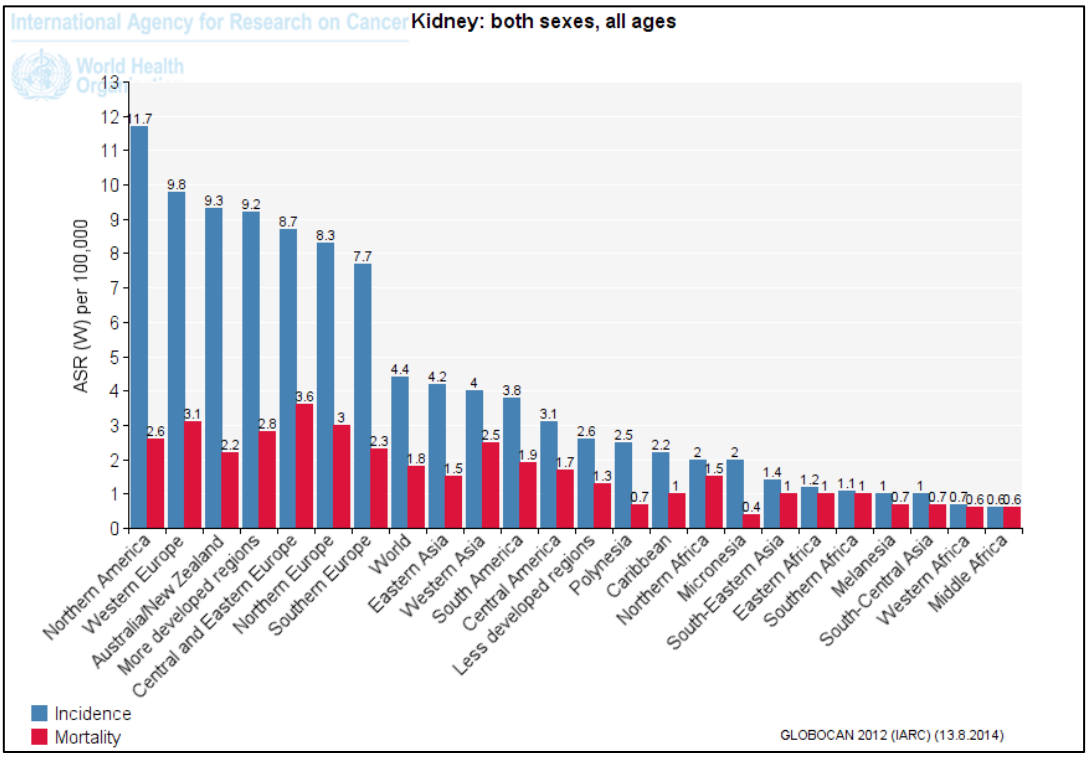


Figure 3 - Distribution of incidence and mortality rates of kidney cancer by 100 000 habitants (Globocan 2012, IARC).

The exact causes of RCC aren't completely identified but epidemiologic studies reveal that certain lifestyle habits seem to be involved in the etiology and development of this disease. Smoking habits, obesity, acquired cystic disease and family history of RCC are well established as risk factors [24, 25]. However, there are others, like nutritional factors, hypertension and occupational exposure, that also seem to modulate the development of RCC but further studies are necessary to clarify their association with the disease [26].

Currently, most RCCs are detected accidentally as a consequence of imaging examinations, such as computed tomography and magnetic resonance imaging, mostly due to symptoms that are not specifically related to RCC [27]. An explanation for this fact is that many renal masses remain asymptomatic and non-palpable until the late stages of the disease [18, 19]. In these cases the prognosis is good and further exams, such as biopsies and aspiration cytology, are needed to confirm the tumor neoplastic nature. Clinical symptoms, such as flank pain, gross heamaturia, palpable abdominal mass, and paraneoplastic syndromes, only appear when the tumor presents a larger diameter, which indicates a worse prognosis [19, 28-30]. This reality and the nonexistence of a standard screening test, contributes to the fact that one-third of patients are diagnosed with local invasive disease or metastatic disease [27]. For patients with clinically localized disease, the 5-year survival rates range from 90.4% for patients with organ-confined disease to 61.7% for patients with regional spread [31]. Regarding the patients that are diagnosed with organ confined disease, 20-40% will also develop metastasis [27]. In patients with distant metastases, the 5-year survival is 9.5% [31].

According to the World Health Organization (WHO), the three major histologic subtypes of RCC are: clear cell (80-90%), papillary (10-15%) and chromophobe (4-5%) and the most aggressive is the clear cell Renal Cell Carcinoma (ccRCC) subtype [32]. Histologic factors influencing prognosis include Fuhrman grade, histologic subtype, presence of sarcomatoide features, microvascular invasion, tumor necrosis, and collecting system invasion. Although it is subject to intraobserver and interobserver discrepancies, the Fuhrman nuclear grade is the most widely accepted histologic grading system for RCC [32]. This classification system groups the neoplasias in four grades of aggressiveness, by evaluating the size and characteristics of the cells nucleous (Table I).

Table I – Fuhrman nuclear grade classification.

	Nuclear diameter	**Nuclear Shape**	**Nucleoli**
Grade I	10 µm	Round, uniforme	Non-visible
Grade II	15 µm	Some irregularities in outline	Visible at high magnification (400X)
Grade III	20 µm	Obvious irregularities in outline	Visible at low magnification (100X)
Grade IV	As Grade III	Bizarre, multi-lobed, with cromatin clumps	Visible at low magnification (100X)

Surgical intervention is the primary treatment for the treatment of RCC detected at early stage. However, surgery alone has a limited benefit in patients with metastatic disease, except for palliative reasons [20, 33]. Until the past decade, the treatment options for patients with metastatic RCC (mRCC) have been extremely limited, as RCC is notoriously resistant to cytotoxic chemotherapy and radiotherapy [34, 35]. Prior to the use of antiangiogenic agents, systemic treatment options for mRCC were limited to cytokine therapies (interleukin [IL]-2 and interferon-alpha [IFN-α]) but they were proved to be ineffective since only a small percentage of the patients showed benefit in long term disease-free survival [36, 37]. In the past years, following the elucidation of various molecular pathways in RCC, targeted agents such as receptor tyrosine kinase inhibitors (TKIs), vascular endothelial growth factor (VEGF) antibodies, and mammalian target of rampamycin inhibitors (mTORs) are now a crucial part of most therapeutic strategies for patients with mRCC [27, 33, 38]. Although the outcome of the patients has improved, many tumors develop resistance to targeted therapies due compensatory change within the target pathway that bypass the site of inhibition [39, 40].

1.2.1. Molecular biology of RCC

As previously said, the three major types of RCC are clear cell (80-90%), papillary (10-15%) and chromophobe (4-5%) [32]. Both the clear cell and the papillary type can be sporadic or caused by inherited syndromes (Figure 4), while the chromophobe type is only caused by an inherited syndrome called Birt-Hogg-Dubé syndrome. This syndrome is a rare autosomal dominant disorder characterized by hair follicle hamartomas (fibrofofolliculomas) of the face and neck, and about 15% of the affected patients have multiple renal tumors (Figure 4) [41, 42].

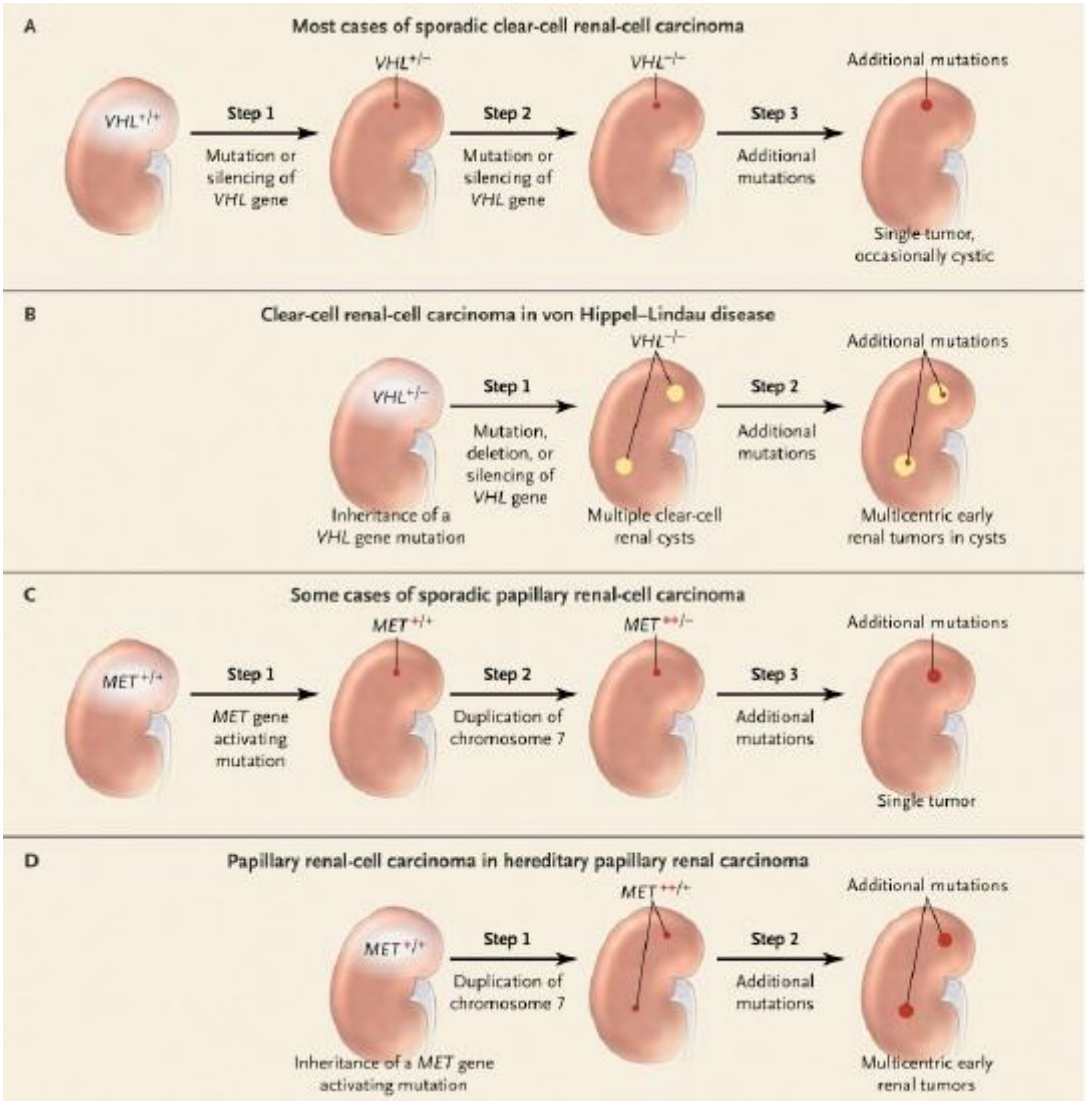


Figure 4 - Steps in the Development of Renal Cell Carcinoma. In contrast to sporadic renal cell carcinoma (A and C), fewer steps are required for the development of RCC in the inherited forms of the disease (B e D), because all of the patient's cells have a mutation that predisposes the patient to the disease. As a result, the disease associated with the familial syndromes occurs earlier and its often multifocal. Each familiar renal cancer syndrome is autosomal dominant. In von Hippel-Lindau (VHL) disease, a cellular recessive mechanism is involved, since both copies of the *VHL* gene are inactivated (A and B). *VHL* is a classic tumor suppressor gene. In hereditary papillary renal carcinoma, one copy of the *MET* gene has an activating mutation, which is inherited (D). Chromosome 7, which includes the defective MET allele, becomes duplicated, increasing the level of expression of the activated MET protein, which is as receptor tyrosine kinase for hepatocyte growth factor. Activated *MET* is a classic oncogene. The (+) represents the wild-type allele; (-) represents a null allele. A (+) in red represents a mutated, activated allele; (+ +) in red represent duplication of that allele. (Adapted from Cohen *et al*. 2005 [35]).

Despite intensive efforts, the molecular mechanisms involved in ccRCC development and progression remain not completely understood. One of the established signaling pathways involved in the pathophysiology of RCC is the von Hippel-Lindau (VHL) pathway [43-46]. In normoxic conditions, the protein encoded by the *VHL* gene serves as a recognition site for the regulatory subunits of HIF, targeting them to the

proteasome degradation (Figure 5 (A)). One of the early molecular events of RCC is the loss of *pVHL* (a consequence of the loss of the short arm of chromosome 3), which stops the degradation of HIF and leads to its accumulation in the cytoplasm and further migration to the nucleus where it binds to hypoxia-related genes, leading to a hypoxic response from the cell in non-hypoxic conditions. Once activated, these genes are involved in pathways responsible for the development of blood vessels, proliferation, glucose metabolism, pH regulation and metastatic disease (Figure 5B) [46-49].

One of the activated genes is Transforming Growth Factor α (TGF-α), which is involved in the induction of cellular proliferation by activating the Epidermal Growth Factor Receptor (EGFR) [50]. The EGFR activates several signaling pathways such as MAPK/ERK and PI3K/AKT which in turn modulate genetic transcription, stimulating cellular proliferation, migration, invasion, angiogenesis and apoptosis (Figure 5C)[51].

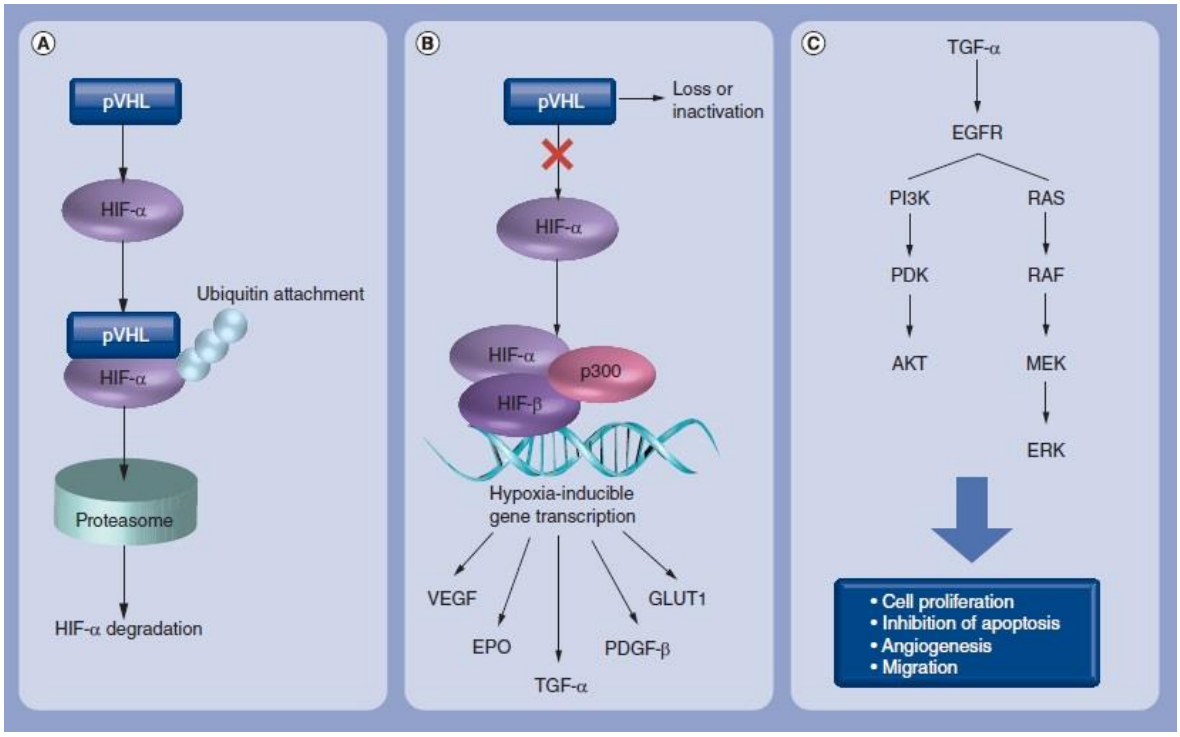


Figure 5 - Signaling pathways involved in renal cell carcinoma pathophysiology. (A) pVHL pathway under normoxic conditions: pVHL targets HIF-α for degradation in the proteasome.(B) pVHL pathway under hypoxic conditions: the loss of VHL leads to the accumulation of HIF-α in the nucleous and consequene binding to transcription factors, which triggers a hypoxic response from the cell. (C) EGFR pathway activation by TGF-α and its consequences. (Adapted from Dias et al. 2013 [49]).

EGFR is a well known tyrosine kinase receptor often deregulated in cancer. This receptor is overexpressed in, approximately, 30% of primary tumors of several cancer types and has been associated with advanced disease, poor prognosis, limited overall survival and therapy response[52, 53].The VHL is also responsible for EGFR turnover and further degradation in the proteosome, thus during the RCC development this process

could be compromised leading to an increase of EGFR [54]. Zhou and co-workers described EGFR half-life as approximately 1 hour in 786-VHL cells but approximately 3 hours in 786-mock cells and that both phospo-AKT and the phospo-ERK signals lasted longer in 786-mock cells than in 786-VHL cells when stimulated with Epidermal Growth Factor (EGF) [54]. It as also been described that hypoxia upregulates EGFR and prolongs its activation through its retention in endocytic trafficking [24]. Recently, Shen and colleagues demonstrated that EGFR is upregulated during tumor progression and specifically enriched in hypoxic tumor areas. They also desmonstrate that EGFR suppresses the maturation of specific tumour-suppressor-like miRNAs in response to hypoxic stress through phosphorylation of argonaute 2 (AGO2) at Tyrosine 393 [55].

If we relate the VHL loss with the consequent increase in EGFR half-life (which is also upregulated by the hypoxic conditions) and TGF-α production, it becomes clear the relevance of studying this pathway to further understanding of ccRCC molecular biology, and the involvement of this pathway in ccRCC tumor progression.

In recent years, the increasing knowledge of the pathways involved in ccRCC has allowed the development of new targeted therapies. The identification of alterations in VHL gene in ccRCC led to the development of targeted therapies such as sunitinib and sorafenib (tyrosine kinase inhibitors) as well as pazopanib (angiogenesis inhibitor) [46, 56]. However, a subset of patients (~25%) do not seem to experience any clinical benefit from these targeted therapies, while in the majority of cases, patients respond to therapy initially but later develop resistance to it and enter in the phase of disease progression [39]. Usually, resistance to the targeted agents in ccRCC patients has been shown to develop after a median of 5-11 months of treatment and a small subset of patients do not experience any clinical benefit from the targeted therapy [40]. These “failures” of targeted therapies show us the necessity of further investigation into the molecular pathways involved in RCC in order to improve our understanding about the molecular events in this type of cancer and to allow for the development of new effective targets and therapies. Another issue related to RCC is the absence of accurate biomarkers for disease diagnosis, follow-up and monitoring treatment response.

Currently, no standard approaches to biomarker sampling or analysis have been adopted for RCC since many of the putative tumor markers themselves are still under active investigation for further validation [57]. The ideal biomarker must be accessible using non-invasive protocols, inexpensive to quantify, specific to the disease of interest, a reliable early indication of disease before clinical symptoms appear and a way to stratify the disease and assess response to therapy [58]. The establishment of a biomarker isn’t an easy task since possible biomarkers in body fluids are susceptible to degradation by circulating proteases and nucleases, thereby decreasing their signal, and on the other

hand, endogenous production of biomarker molecules by normal cells may artificially augment the signals [59, 60].

Urine metabolomics analysis is theoretically promising but difficulties with the heterogeneous nature of urine metabolites, potential contamination of nonhuman metabolites from genitourinary flora, and special handling requirements have limited the progress of using it as a source of biomarkers in RCC [61]. Although many groups have studied biomarkers for diagnosis, prognosis and prediction of therapy response in RCC, these efforts have unfortunately been preliminary and uncoordinated. The majority of the studies were limited by small, retrospective, convenience cohorts that have not been replicated by independent groups while others have been validated with small test subsets and performed well, but they also lack external validation with larger cohorts [57].

From all the possible biomarkers that were studied in RCC, the ones that can fit all the categories (diagnosis, prognosis and predictive of response) were the miRNAs, but as the rest, further research is needed in order to validate them [62-66].

1.3. MicroRNAs

MiRNAs are a family of small non-coding RNAs (19-25 nucleotides in length) that regulate gene expression by sequence-selective targeting of mRNAs, leading to their degradation or blockade at the post-transcriptional level, depending on the degree of complementarity between miRNAs and the target mRNA sequence [67, 68]. For the majority of miRNAs, the primary transcripts are generated in the nucleus [69]. Following transcription, the pre-miRNA is processed by DROSHA and its binding partner DGCR8 (also known as Pasha), creating a pre-miRNA [70]. The pre-miRNAs are exported to the cytoplasm by the nuclear export protein XPO5, where they are further processed by DICER, leading to the production of mature 22 nucleotide-stranded molecules [71]. The mature miRNA enters the RISC complex where it becomes a single-stranded functional miRNA. This single-stranded miRNA then induces post-transcriptional gene silencing by guiding the RISC complex to target miRNAs. Target recognition occurs mainly by incomplete base pairing complementarity between the miRNA and the target mRNA resulting in mismatches that, in turn, lead to target gene silencing, which can occur via translational repressing or mRNA degradation (Figure 6) [71, 72].

MiRNA expression is dynamic, since it is postulated that each miRNA regulates up to 100 different mRNAs and that more than 10.000 mRNAs appear to be directly regulated by miRNAs [73]. This variability makes miRNAs potent modulators of cellular behavior,

since several miRNAs can target the same gene and one single miRNA can target multiple genes [74]. As a single miRNA may target up to several hundred mRNAs, aberrant miRNA expression may affect a multitude of transcripts and profoundly influence cancer-related signaling pathways [69]. Hence, miRNAs have been identified as key regulators in many biological processes including cell development, differentiation, apoptosis and proliferation [75]. Many miRNAs have been identified to act as oncogenes, tumor suppressors or even modulators of cancer stem cells and metastasis formation [76]. OncomiRs are known to downregulate tumor suppressor genes, and have been reported to be overexpressed in multiple miRNA-profiling studies. On the other hand, tumor suppressor miRNAs are responsible for downregulating oncogenes, and are mostly underexpressed in cancer [33].

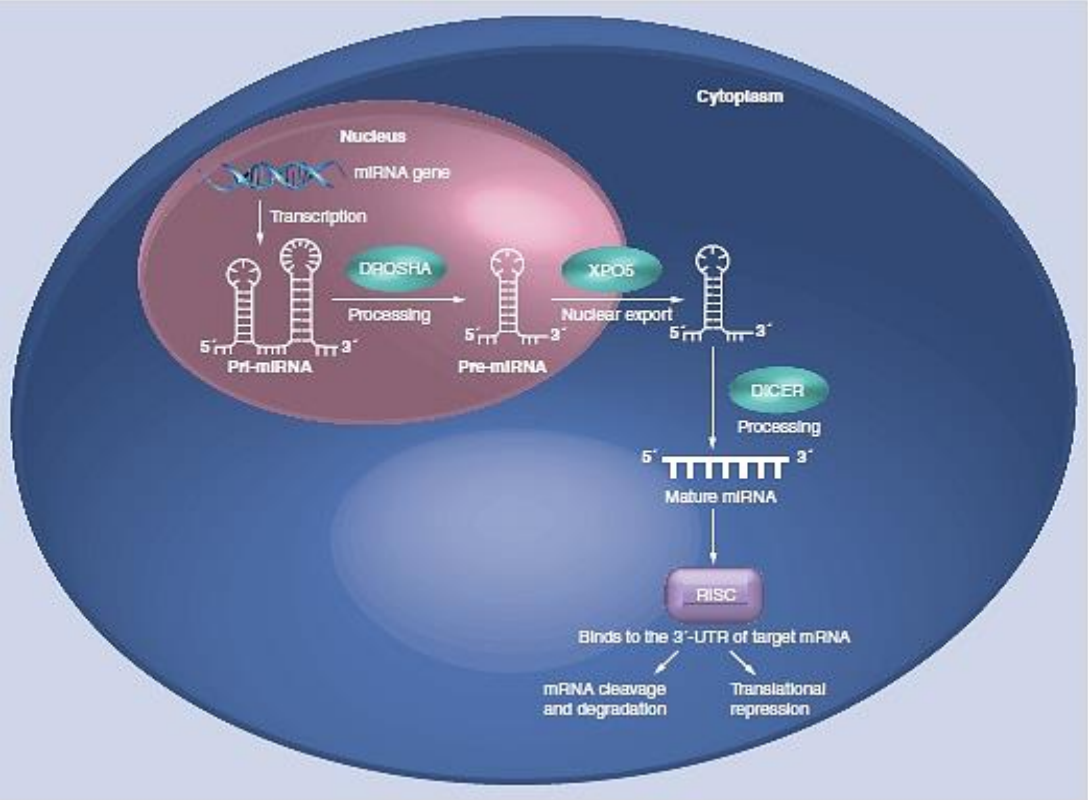


Figure 6 - MiRNA biogenesis and function (adapted from Dias *et al*. 2013 [49]).

One of the most important features of miRNAs is that they have different expression patterns in normal cells when compared with cancer cells, which makes them excellent candidates for biomarkers [77]. In addition to that, miRNA expression signatures in blood, serum and plasma are similar between species, as well in individuals of different ages [58]. On the other hand, levels of miRNAs in circulating samples are reproducible and consistent among individuals from the same species and specific expression patterns

of serum miRNAs have already been identified for pregnancy, diabetes, and different cancers, thus providing evidence that plasma miRNAs contain fingerprints distinctive of certain human conditions [19]. Circulating miRNAs are also stable after being submitted to severe conditions, such as boiling, very low or high pH, extended storage, and several freeze-thaw cycles, conditions that would normally degrade most RNAs [78]. But the most interesting characteristic about circulating miRNAs is that they seem to be protected from RNase activity, which solves the problem of possible degradation and launches them as the one of the top candidates for circulating biomarkers. One of the possible explanation for their elevated stability in circulating samples is that they circulate in exosomes [13]. As previously explained (Figure 2), exossome membranes are enriched in cholesterol, sphingomyelin, and ceramide as well as lipid raft associated proteins [79, 80]. These components allow exosomes to be highly stable and thus be collected from numerous body fluids including blood, urine, breast milk, ascites and saliva [81-86]. Consistent with this theory is that several authors reported differences in miRNA content when comparing exosomes from normal individuals with cancer patients [87-89]. Although normal cells within the peripheral circulation can contribute to exossome population, the primary source of circulating exosomes in cancer patients is the tumor [90]. Moreover, miRNA containing tumor-derived exosomes can affect biological processes inside of recipient cells and, consequently, affect the tumor microenvironment [13]. MiRNA molecules have also been described in exosomes shed from several tumor cell lines, including lung, gliobastomas and gastric cancers [87, 88, 91]. It has also been suggested that tumor derived exosomes could be a vehicle involved in the metastization process. Grange and co-workers found that CD105-positive exosomes (containing miRNAs) that were released by renal cell carcinoma stem cells triggered angiogenesis and the formation of a premetastatic nich in the lungs when injected in mice [92].

The ubiquitous nature of exosomes in body fluids makes them ideal for use in diagnostic and prognostic biomarker studies. Nevertheless, little is known about the mechanisms in which miRNAs are selected and incorporated in exosomes and the biological impact of these molecules in distant sites of the body [93].

1.3.1. MicroRNAs as potential biomarkers in RCC

The majority of the efforts made with the purpose of finding a signature of deregulated miRNAs in RCC has been made mainly through microarray analysis or next-generation deep sequencing analysis, and focusing on tissue samples [94-97]. Since microarray analysis allows the expression analysis of hundreds of miRNAs at the same time, it can be an easy way to get an overall view of the overexpressed and underexpressed miRNAs in RCC. However, these analysis are made using a limit number of patients mainly due to the fact that to obtain the tissue samples invasive methods, such as biopsies, are required. Nevertheless, some advances have been made. Youssef and coworkers developed a classification system, using miRNA analysis and tissue samples, which can distinguish the different RCC subtypes using unique miRNAs signatures in a maximum of four steps. The system has a sensitivity of 97% in distinguishing normal RCC, 100% for clear cell RCC subtype, 97% for papillary RCC subtype and 100% accuracy in distinguish oncocytoma from chromophobe RCC subtype [77].

Regarding the circulating miRNAs in RCC, only a few have been suggested as potential biomarkers. MiR-378 which is known to promote cell survival and angiogenesis and miR-451 are upregulated in serum of RCC patients, and combined enable the identification of RCC serum with the sensitivity of 81% and specificity of 83% [63]. MiR-210, which is induced by HIF-1α, is also upregulated in serum of RCC patients compared to healthy controls with a sensitivity of 81% and a specificity of 79% [66]. MiR-1233, which seems to be involved in HIF-1α activation, is also increased in the serum of RCC patients, with a sensitivity of 77.4% and a specificity of 37.6% [62].

Despite promising, these miRNAs were studied in small cohorts and need replication in larger samples for further validation as circulating biomarkers in RCC. Since the EGFR pathway is an important pathway in the molecular biology of RCC, the studying of some miRNAs involved in it, such as the cluster miR-221/miR-222, which is induced by EGFR activation, or miR-218 which is a tumor suppressor miRNA that targets EGFR, seem good candidates to add to some of the previous studied circulating miRNAs [98-101]. Thus we can elucidate the role of this pathway in RCC, expand the study of circulating miRNAs and try to establish a miRNA signature that could act as a biomarker of diagnosis and/or prognosis (Figure 7).

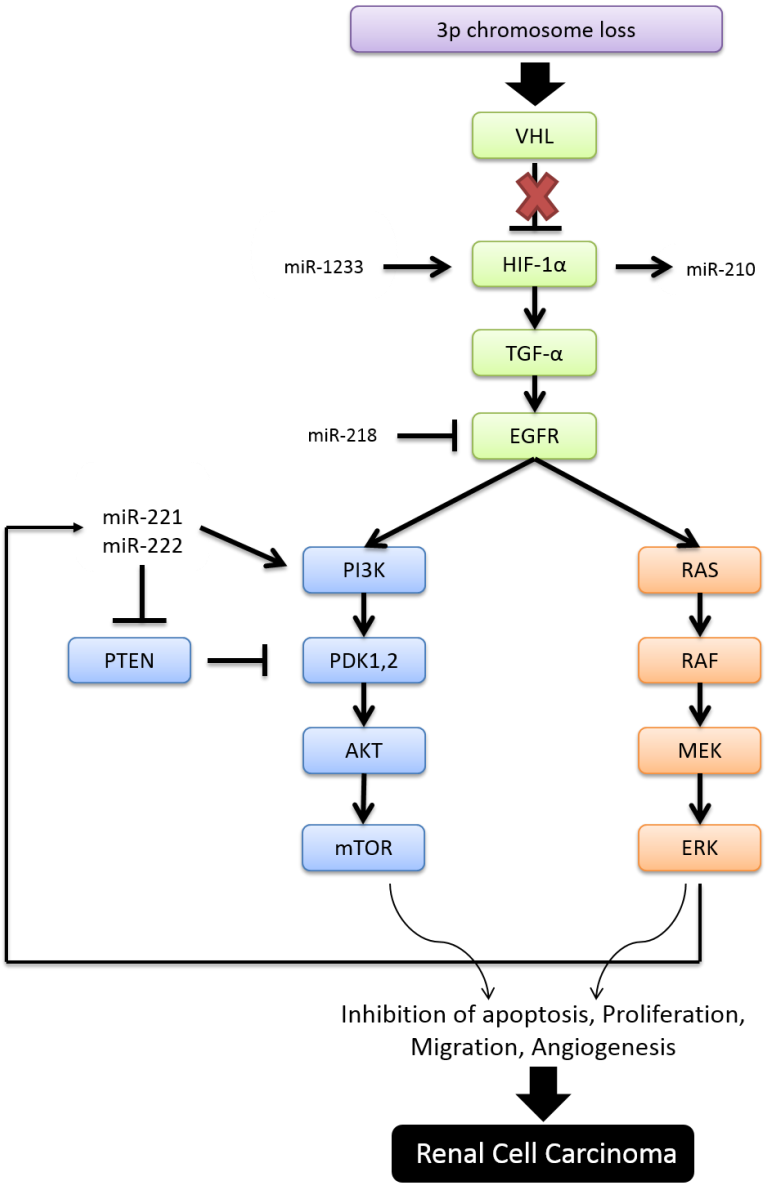
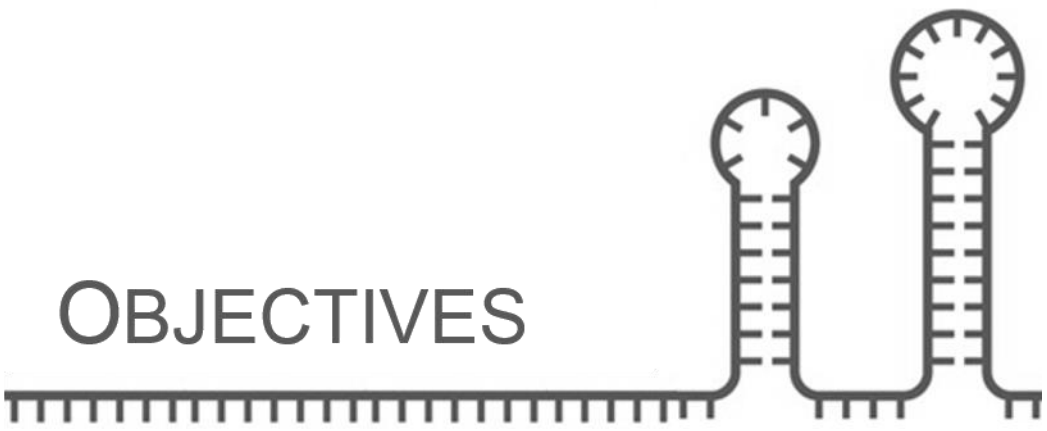


Figure 7 - Representative model of VHL and EGFR pathways under hypoxic conditions and the proposed roles of miR-210, miR-218, miR-221, miR-222 and miR-1233 in RCC development.

OBJECTIVES



2. Objectives

2.1. Main objective

The aim of the present work is the establishment of a circulating miRNA expression profile that could be used as biomarker of diagnosis and/or prognosis in RCC patients.

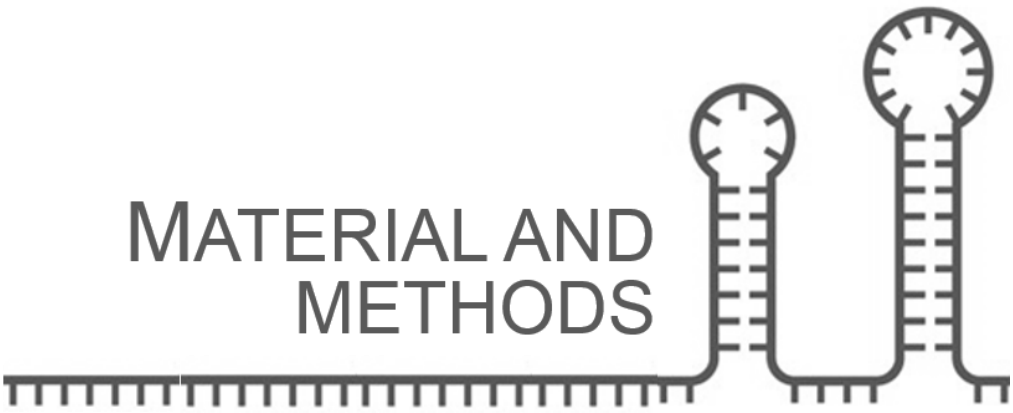
2.2. Specific objectives

- In a first phase it will be performed an *in vitro* study using a normal renal cell line and a renal cell carcinoma cell line to establish a circulating miRNA profile associated with RCC development

- In a second phase it will be performed an i*n vivo* study using plasma samples of healthy individuals *versus* RCC patients in order to validate the previous established miRNA expression profile

- Evaluation of the overall survival in RCC patients according to the established miRNA expression profile

MATERIAL AND METHODS



3. Material and Methods

3.1. Phase I: *In vitro* study

3.1.1. Cell line characterization

For this study, two renal cell lines were used: HKC-8 and FG-2. The HKC-8 cell line is described as proximal tubular normal renal cell line (Figure 8A). The FG-2 cell line is described as a metastatic RCC cell line (Figure 8B). Both cell lines were kindly provided by Dr. Klaas Kok from Groningem University, Netherlands.

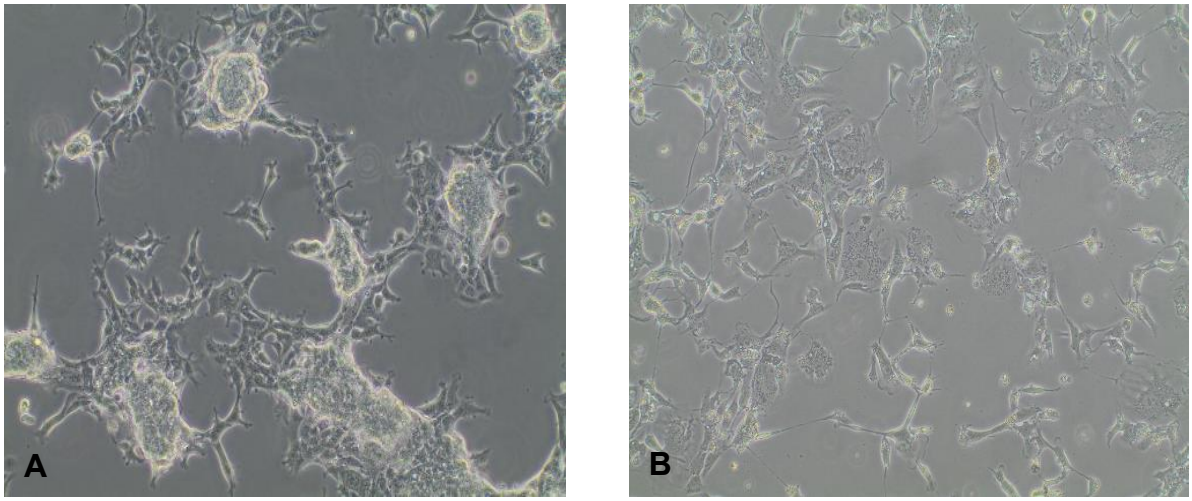


Figure 8 - (A) Microscopic image (10X) of the HKC-8 cell line. (B) Microscopic image (10X) of the FG-2 cell line. (Both photographs were taken using an Olympus IX51 microscope).

To confirm the tumoral phenotype of the cell lines, it was performed a Fluorescence *in situ* Hybridization (FISH) technique in both cell lines to detect the presence (or absence) of the *VHL* gene. It was used a specific probe for the centromere of the short arm of chromosome 3 and a specific probe for the *VHL* gene. As expected there were the same number of centromeres of chromosome 3 and respective *VHL* genes in the HKC-8 cell line (Figure 9A) and loss of the *VHL* gene in the FG-2 cell line (Figure 9B).

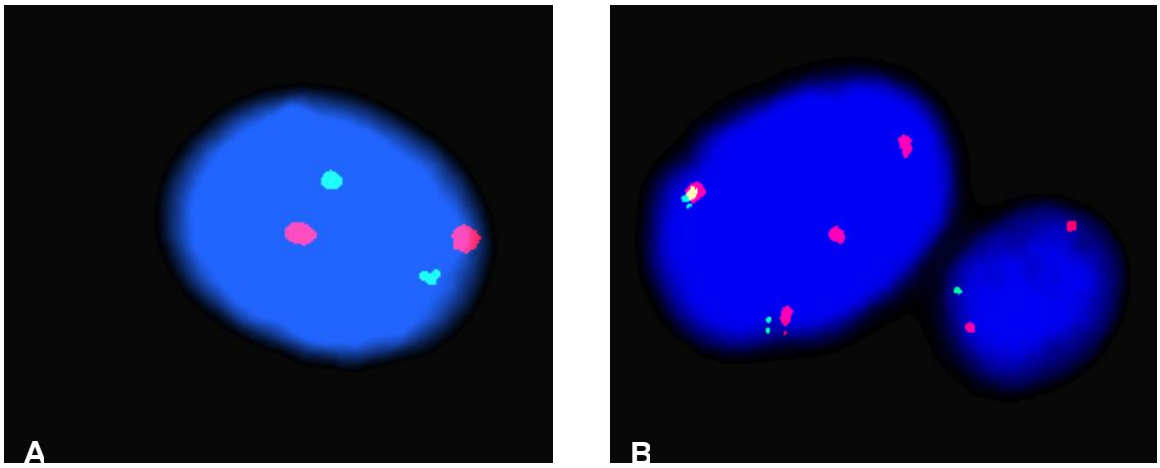


Figure 9 - (A) Microscopic image of the chromosome 3 centromeres and VHL genes present in the HKC-8 cell line. (B) Microscopic image of the chromosome 3 centromeres and VHL genes present in the FG-2 cell line. (Red dye - chromosome 3 centromere; green dye – VHL gene). Images provided by Dr. Joana Vieira from the IPO-Porto Genetic Department.

3.1.2. MicroRNA extraction and cDNA synthesis

Initially a cryopreserved vial of each cell line was thawed. The FG-2 cell line was mantained in RPMI 1640 (1X) medium (*Gibco*®), supplemented with 10% of FBS (Fetal Bovine Serum) (*Gibco*®), and 1% of Pen-Strep (*Gibco*®). The HKC-8 cell line was kept in DMEM/F12 medium (*Gibco*®), supplemented with ITS (Insuline-transferrine-selenium) (*Sigma-Aldrich*®), Pen-Strep (*Gibco*®), EGF (Epidermal Growth Factor) (*Sigma-Aldrich*®), Hepes buffer (*Gibco*®) and Hydrocortisone (*Sigma-Aldrich*®). Both cell lines were mantained in a 5% CO_2 incubator at 37ºC.

When the desired confluence was achieved (80-90%) the medium, in which the cells were being cultured, was collected for miRNA extraction and the cells were trypsinized , using 0.05 % trypsin-EDTA (1×) (*Gibco*®) and counted using a Neubauer chamber and Tripan-Blue dye (*Gibco*®). After counting, approximately two million cells were centrifuged to form a pellet for miRNA extraction and the remaining cells were kept in culture.

This procedure was repeated five times for each cell line and respective medium.

3.1.3. MicroRNA extraction and cDNA synthesis

MicroRNA extraction (from the cells and respective medium) was performed using the *GRS microRNA kit* (*Grisp*®) according to manufacture instructions. After isolation,

miRNA concentration and purity were measured at 260 and 280 nm using the *NanoDrop® ND-1000* spectrophotometer. The miRNA samples were then used as templates for cDNA synthesis using a *Taqman®MicroRNA Reverse Transcription kit* (*Applied Biosystems®*) and sequence-specific stem-loop primers for miR-210, miR-218, miR-221, miR-222, miR-1233 and RNU-48. After protocol optimization the thermal conditions were as follows: 16ºC for 30 min, followed by 42ºC for 60 min and 85ºC for 10 min.

3.1.4. Real-time PCR relative quantification

The miRNA expression was analyzed by quantitative real-time PCR. The reactions were carried out on a StepOne™qPCR Real-Time PCR machine, containing 1X Master mix (*Applied Biosystems®*), with 1X probes (*TaqMan® microRNA Expression Assays* , miR-210:TM000512, miR-218:TM002094, miR-221: TM-002096, miR-222: TM-002097, miR-1233:TM-002768, *Applied Biosystems®*), cDNA sample and RNU-48 endogenous control (*TaqMan® Gene Expression Assays*, TM-001006, *Applied Biosystems®*) which was used to normalize the results, regarding the biomarkers, since it presents a constant expression level. Data analysis was made using StepOne™ Sofware v2.2 (*Applied Biosystems®*) with the same baseline and threshold set for each plate, in order to generate threshold cycle (Ct) values for all the miRNAs in each sample. The miRNAs quantification was performed in duplicate and negative control lacking cDNA was included in all reactions. The results were confirmed by two independent investigators. The $2^{-\Delta\Delta Ct}$ method (Livak method) and the t' Student test were used to evaluate the differences in the expression levels of the normalized miRNAs.

This procedure was made 5 times for each cell line and respective medium.

3.2. Phase II: *In vivo* study

3.2.1. Study population

The validation of the miRNA profile was made through a case-control study, involving a total of 100 individuals (50 RCC patients and 50 healthy individuals). All RCC individuals were Caucasian from the north of Portugal, with histopathologic diagnosis of ccRCC, admitted and treated at the Portuguese Institute of Oncology of Porto, 38 were male and 12 female, with ages between 60,4±12,4.

All the demographic and histopathologic data were collected from the clinical files of each patient. The staging of each patient was made according to the AJCC TNM classification system 2006 (6th edition) (Table II). All samples were collected after previous consent of each patient, according to the Helsinky declaration.

Table II – Histopathological characteristics of the population.

	Cases (n = 50)		Control Group (n =50)	
	n	%	n	%
Gender				
Male	38	76	16	30
Female	12	24	34	68
Age				
Average ± SD	60.4 ± 12.4		43.0 ± 15.5	
Median				
Histology				
Clear Cell	36	72		
Others	14	28		
Unknown	-			
TNM Stage				
I – II	19	38		
III – IV	31	62		
Unknown	-	-		
T				
T1	17	34		
T2-T3	33	66		
N				
N0-N2	9	18		
Nx	41	82		
M				
M0	38	76		
M1	12	24		
Fuhrman Grade				
G1-G2	15	30		
G3-G4	32	64		
Unknown	3	6		
Microvascular invasion				
Yes	7	14		
No	34	68		
Unknown	9	18		

For the control group, 50 healthy Caucasian individuals with no history of cancer, were randomly recruited from the north of Portugal. All demographic data were collected through a written form, authorized by the ethics committee of the Portuguese Institute of Oncology of Porto, and signed by each individual, according to the Helsinki declaration.

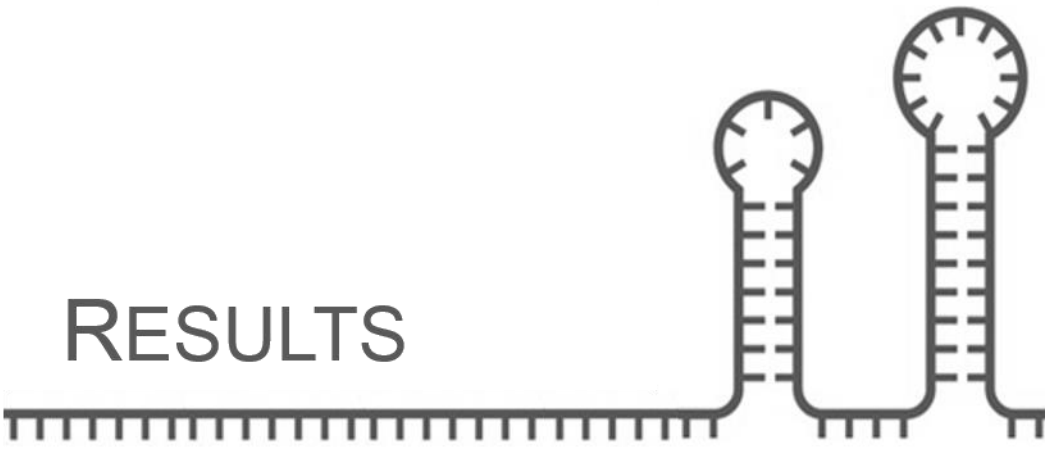
3.2.2. Sample collection and miRNA extraction, purification and quantification

Approximately 8 mL of peripheral blood were collected from all individuals through a standard method of intravenous collection with EDTA tubes. The blood tubes were centrifuged 5 minutes at 3000 rpm in order to separate the plasma from the blood cells. For miRNA isolation, we added an acid phenol-chloroform (5:1) solution (*Ambion*®) to the plasma samples, which after centrifugation at 15,000 rpm for 15 min allows the separation of the RNA phase. Then the miRNAs were isolated through the GRS microRNA kit (Grisp®), the miRNA samples were then used as template for cDNA synthesis and miRNA expression was analyzed by quantitative real-time PCR as previously described.

3.2.3. Statistical Analysis

Statistical analysis was also made using IBM®SPSS®Statistics software for Windows (Version 22.0). The $2^{-\Delta\Delta Ct}$ method (Livak method) and the t' Student test were used to evaluate the differences in the expression levels of the normalized miRNAs. The Kaplan-Meier method and Log-rank test were used to compare the miRNA expression profile influence in the patients overall survival.

RESULTS



4. Results

4.1. Phase I: *In vitro* study

In figures 10 to 14, are represented the graphs relative to the intracellular and extracellular relative expression levels of miR-210, miR-218, miR-221, miR-222 and miR-1233 found in both cell lines included in the *in vitro* study.

According to the results obtained for miR-210, there are no differences in the intracellular levels of this miRNA when we compare both cell lines (*P*=0.324). Regarding miRNA excretion, when we compare the intracellular levels with the extracellular levels of miR-210 in the HKC-8 cell line we can´t observe any differences (*P*=0.744). However, when we compare the intracellular levels with the extracellular levels of miR-210 in the FG-2 cell line, we found a 2.64 fold-increase in the extracellular levels (*P*=0.020). In addition, when we compare the excretion of miR-210 in both cell lines, we observe that the FG-2 cell line presents a 5.94 fold-increase of miR-210 expression levels when compared to the HK-C8 cell line (*P*=0.016) (Figure 10).

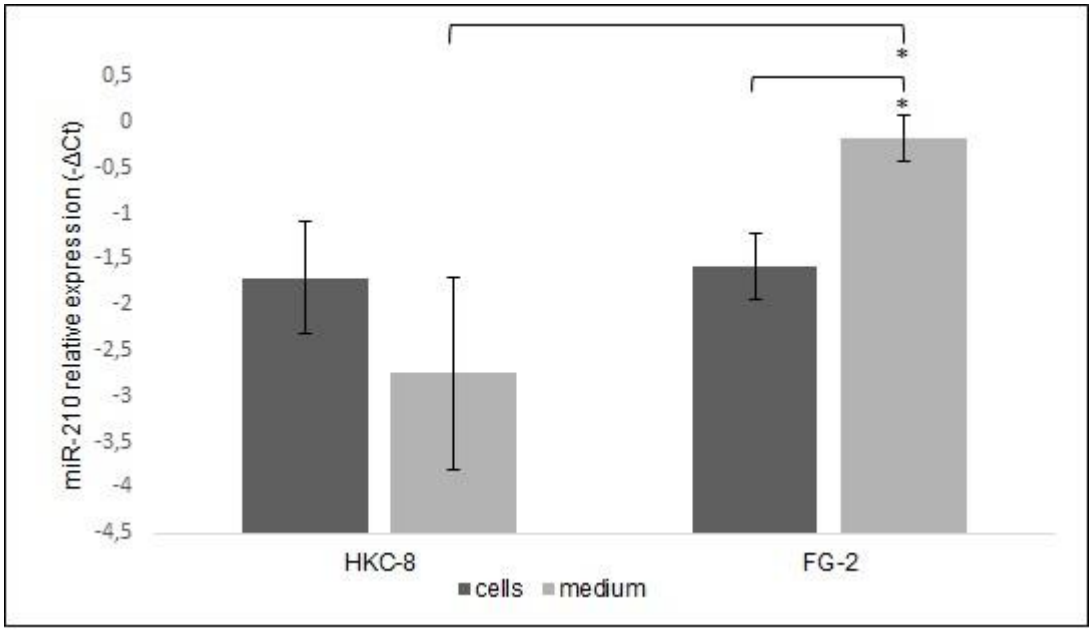


Figure 10 - Variation of the intracellular and extracellular expression levels of miR-210 in HKC-8 and FG-2 cell lines and respective medium (Mean ± Std.Error; *$P \leq 0.050$).

According to the results obtained for miR-218, there are no differences in the intracellular levels of this miRNA when we compare both cell lines (*P*=0.240). Regarding

miRNA excretion, when we compare the intracellular expression levels with the extracellular expression levels of miR-218 in the HKC-8 cell line we can't observe any statistical significant differences (*P*=0.482). Although, when we compare the intracellular expression levels with the extracellular expression levels of miR-218 in the FG-2 cell line, we can see a 302 fold-increase in the extracellular expression levels (*P*=0.002). However, if we compare the extracellular expression levels of miR-218 in both cell lines we don't observe any statistical significant difference (*P*=0.089) (Figure 11).

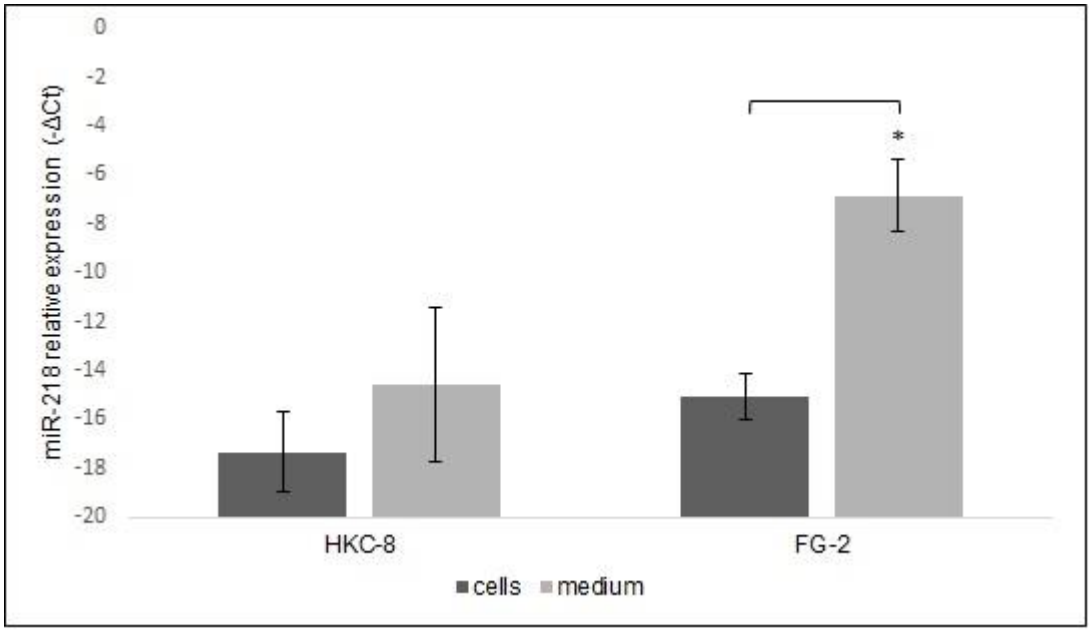


Figure 11 - Variation of the intracellular and extracellular expression levels of miR-218 in HKC-8 and FG-2 cell lines and respective medium (Mean ± Std.Error; **P*≤0.050).

According to the results obtained for miR-221, there are no statistical significant differences in the intracellular levels of this miRNA when we compare both cell lines (*P*=0.213). The same happens when we compare the extracellular levels of miR-221 with intracellular levels in the HKC-8 cell line (*P*=0.674). However, when we analyze the FG-2 cell line we can observe a trend for higher levels of miR-221 in the medium (*P*=0.060). When comparing the extracellular expression levels of miR-221, we also can´t observe any differences between both cell lines (*P*=0.934) (Figure 12).

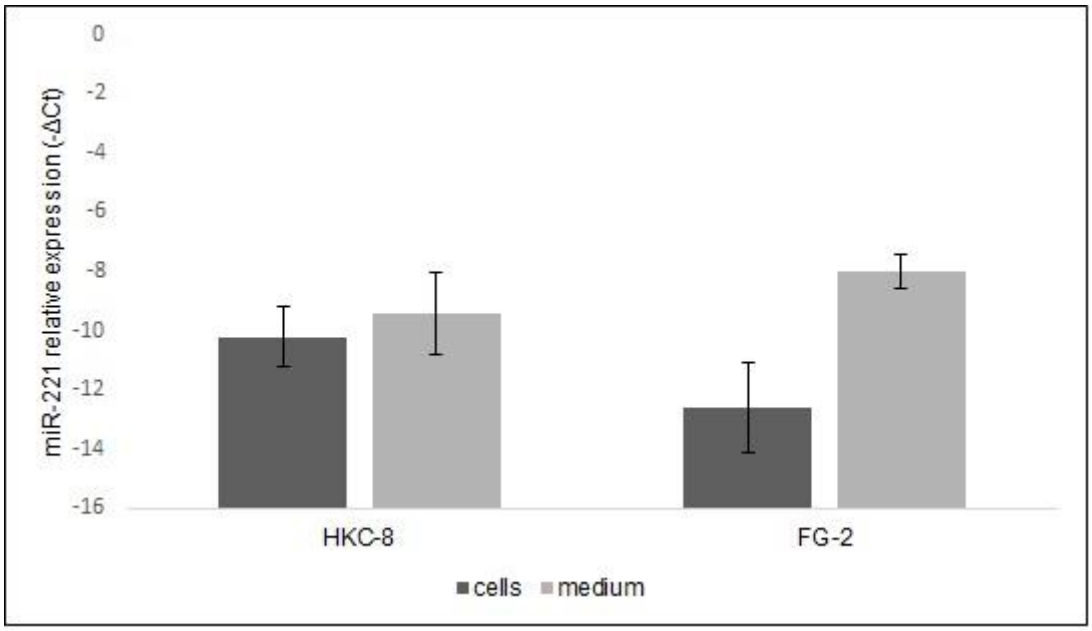


Figure 12 - Variation of the intracellular and extracellular expression levels of miR-221 in HKC-8 and FG-2 cell lines and respective medium (Mean ± Std.Error).

According to the results obtained for miR-222, there are no statistical significant differences in the intracellular expression levels of this miRNA when we compare both cell lines (*P*=0.303). The same happens when we compare the extracellular expression levels of miR-222 with intracellular expression levels in the HKC-8 cell line (*P*=0.342) and in the FG-2 cell line (*P*=0.088). Regarding the extracellular expression levels of miR-222, we also can´t observe any statistical significant differences between both cell lines (*P*=0.759) (Figure 13).

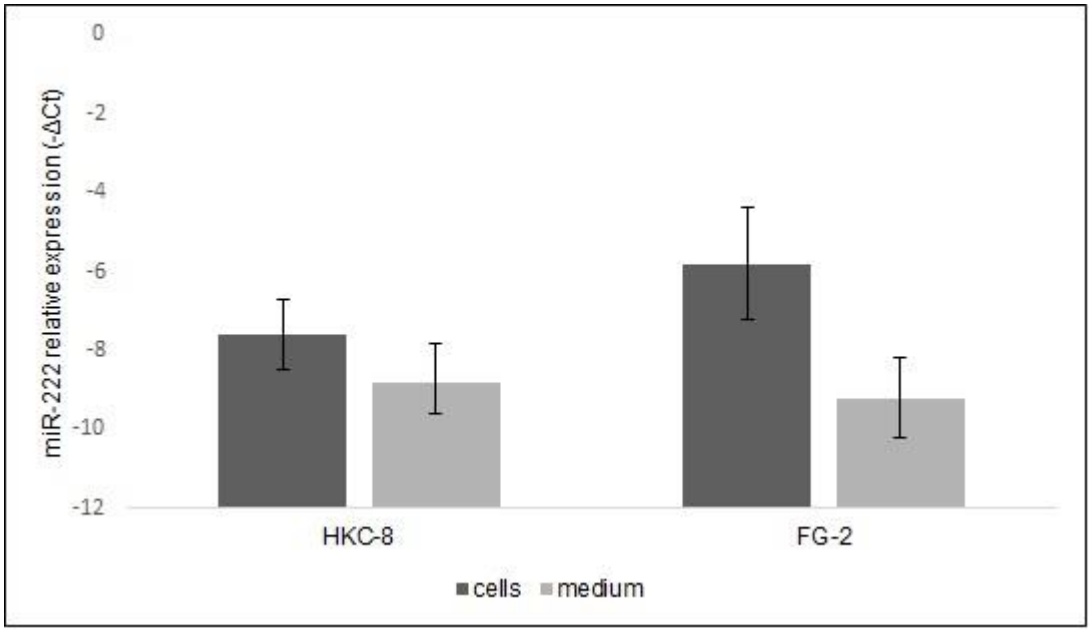


Figure 13 - Variation of the intracellular and extracellular expression levels of miR-222 in HKC-8 and FG-2 cell lines and respective medium (Mean ± Std.Error).

According to the results obtained for miR-1233, there are no statistical significant differences in the intracellular levels of this miRNA when we compare both cell lines (*P*=0.589). Regarding miRNA excretion, when we compare the intracellular expression levels with the extracellular expression levels of miR-1233 in the HKC-8 cell line we can´t observe any statistical significant differences (*P*=0.327). However, when we compare the intracellular expression levels with the extracellular expression levels of miR-1233 in the FG-2 cell line, we can see 10.63 fold-increase in the extracellular expression levels (*P*=0.021). In addition to that, if we compare the excretion of miR-1233 in both cell lines, we can also observe a trend for an increase in the FG-2 cell line (*P*=0.054) (Figure 14).

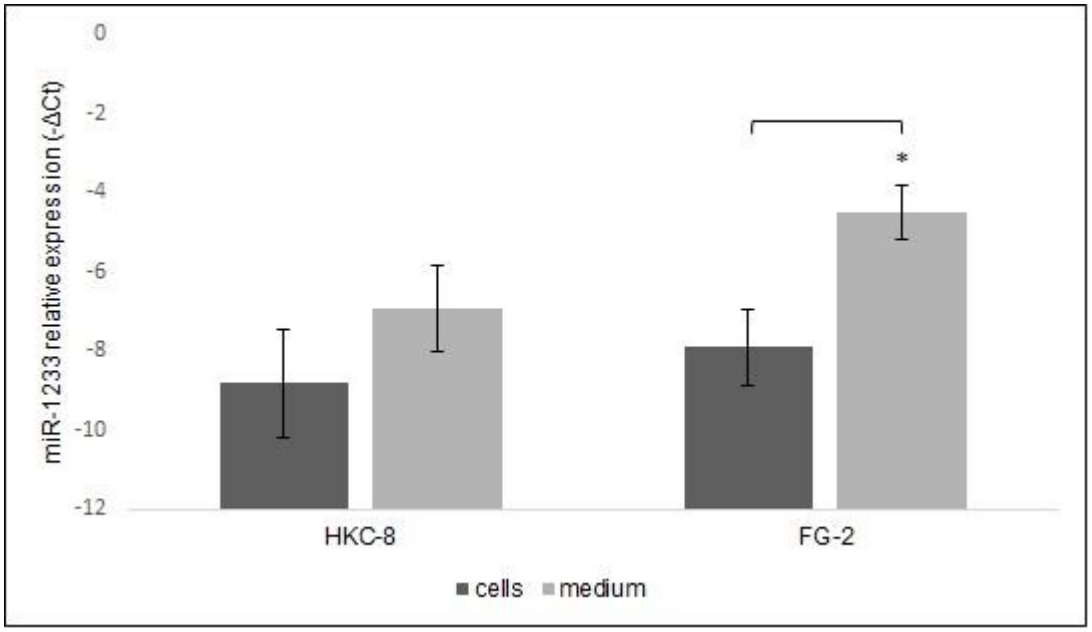


Figure 14 - Variation of the intracellular and extracellular expression levels of miR-1233 in HKC-8 and FG-2 cell lines and respective medium (Mean ± Std.Error; *P≤0.050).

4.2. Phase II: *In vivo* study

According to the in vitro study we observed higher extracellular expression levels of miR-210, miR-218 and miR-1233 in FG-2 cells, thus these miRNA profiles was analyzed in plasma samples of RCC patients.

In figure 15, we can observe a significant increase in the expression levels of miR-210, miR-218 and miR-1233 in the plasma of RCC patients, when compared to healthy controls ($P \leq 0.001$; $P \leq 0.001$; $P \leq 0.001$; respectively). We observed a 5.24; 27.10 and 52.34 fold-increase in miR-210, miR-218 and miR-1233 expression levels, respectively.

In figures 16 to 19 is represented the miRNAs relative expression levels in the plasma of the RCC patients and its relation with clinicopathological characteristics.

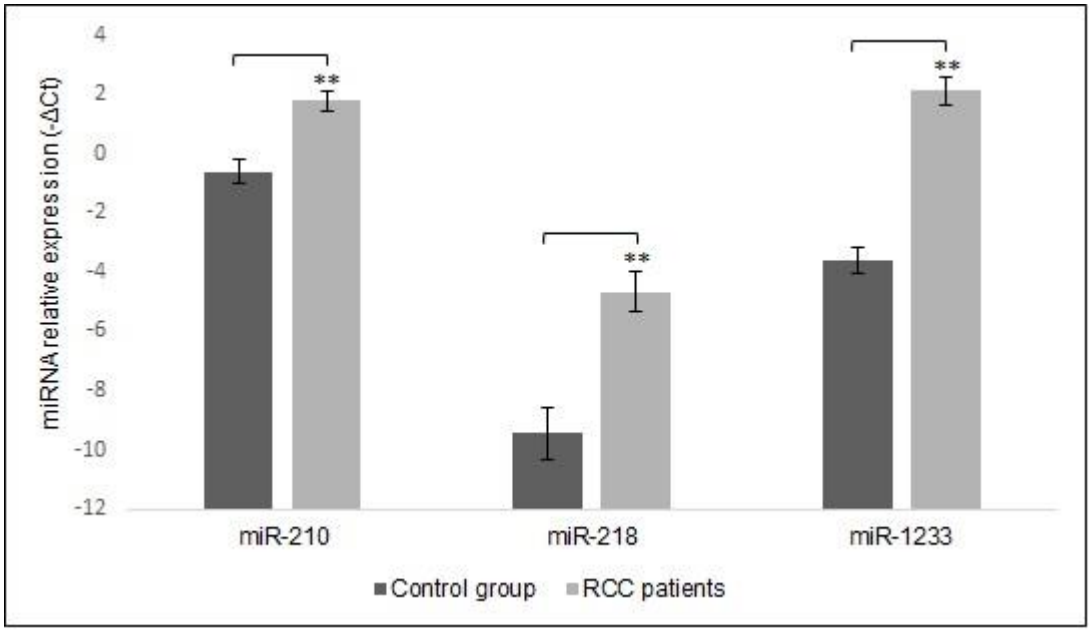


Figure 15 - Relative expression of miR-210, miR-218 and miR-1233 in the plasma of RCC patients compared with healthy controls (Mean ± Std.Error; ** $P \leq 0.001$).

Regarding tumor size, we observed that miR-210 and miR-1233 expression levels were increased in larger tumors ($P \leq 0.001$; $P = 0.007$ respectively) while for miR-218 no significant statistical differences were observed ($P = 0.069$). The fold-increase for miR-210 was 7.84 and for miR-1233 was 8.05 (Figure 16).

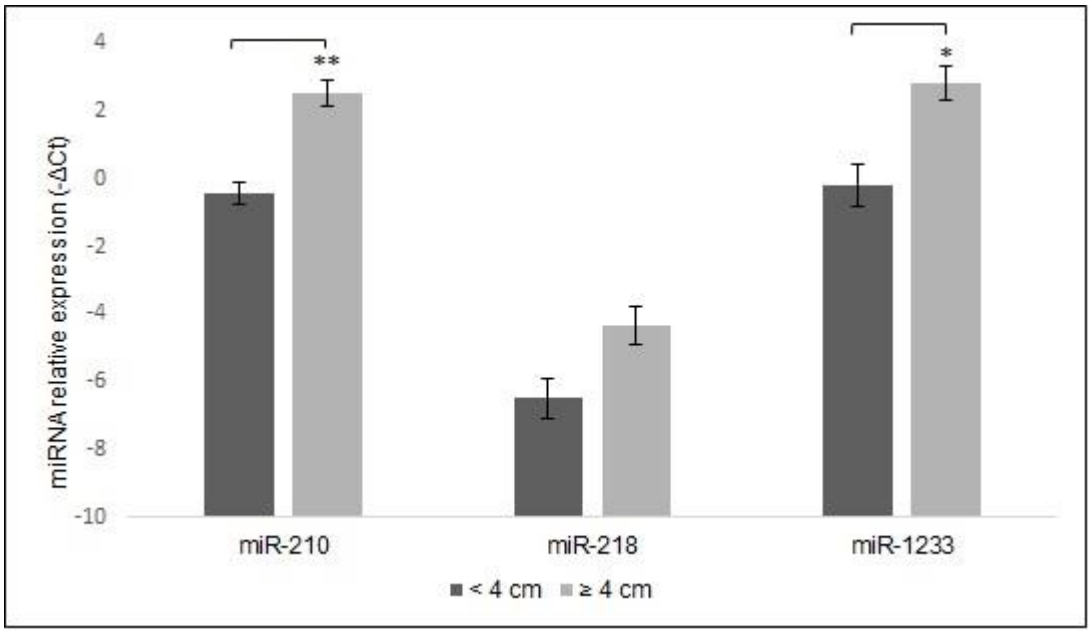


Figure 16 - Relative expression of miR-210, miR-218 and miR-1233 considering tumor size (< 4cm vs ≥ 4cm) (Mean ± Std.Error; **P*≤0.050; ***P*≤0.001).

When we compare the miRNAs expression levels with the Fuhrman nuclear grade, we only observe a 5.13 fold-increase in miR-1233 (P=0.011), (miR-210 P=0.114 and miR-218 P=0.216) (Figure17).

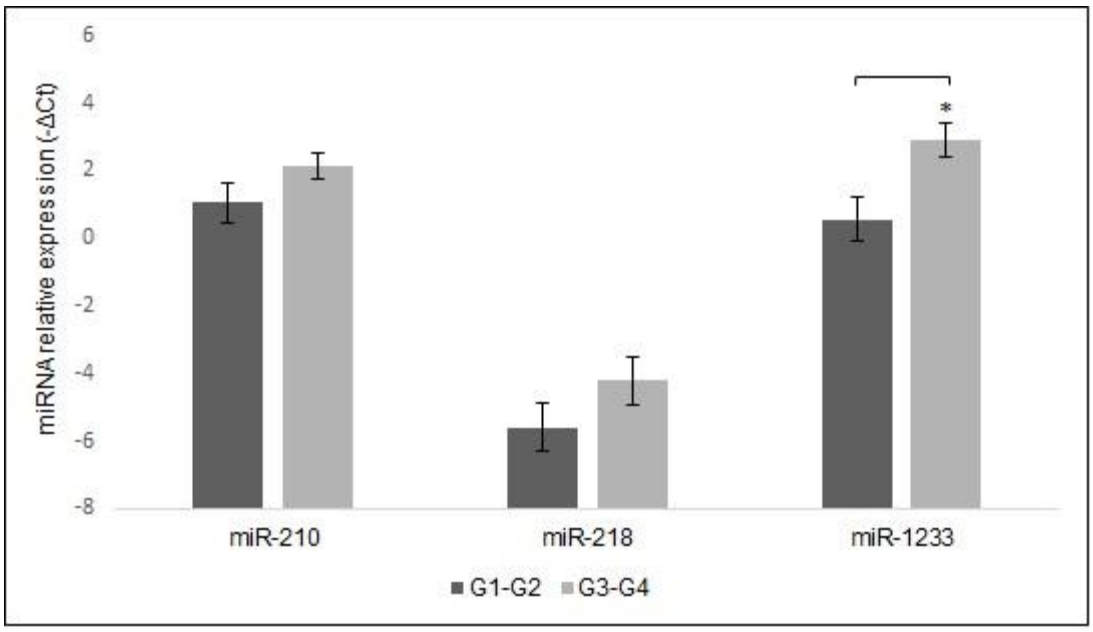


Figure 17 - Relative expression of miR-210, miR-218 and miR-1233 considering the Fuhrman nuclear grade (Mean ± Std.Error; **P*≤0.050).

Regarding the microvascular invasion, we observe an increase in the expression levels of miR-210 (*P*=0.049) and miR-1233 (*P*=0.012) with fold-increase of 3.97 and 8.40, respectively. For miR-218, we didn't observe any significant result (*P*=0.191) (Figure 18).

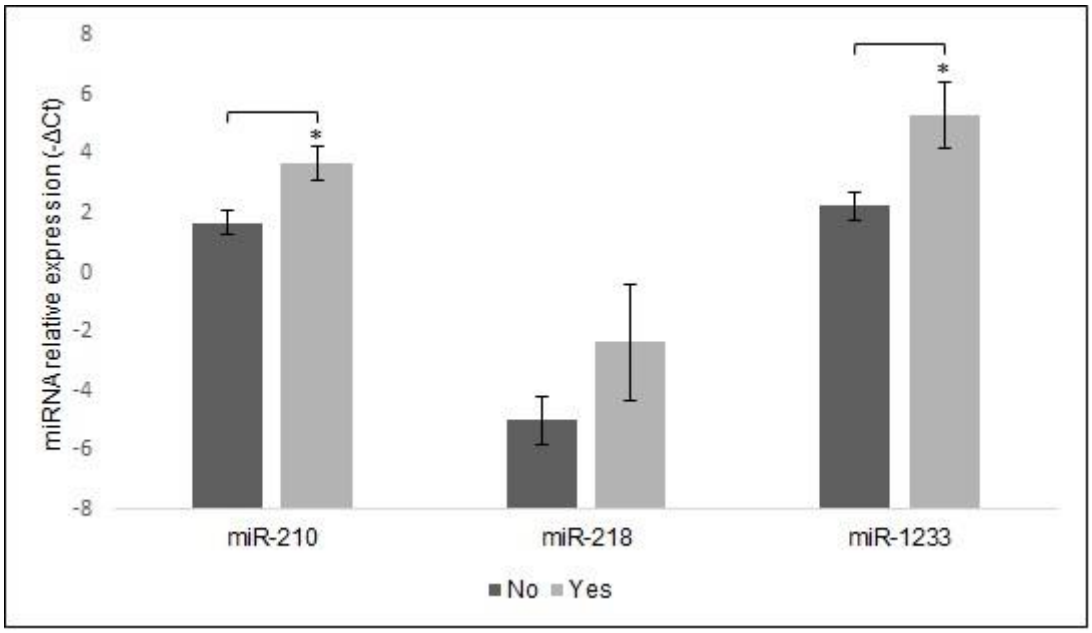


Figure 18 - Relative expression of miR-210, miR-218 and miR-1233 considering microvascular invasion (Mean ± Std.Error; *$P \leq 0.050$).

When analyze the miRNAs expression taking into account the presence of metastasis, we can observe that the levels of all three miRNAs are higher in patients with metastatic disease when compared to patients with local disease (miR-210, $P=0.005$; miR-218, $P=0.014$; miR-1233, $P=0.032$). The fold-change for miR-210 was 4.63, for miR-218 was 16.68 and for miR-1233 was 5.43 (Figure 19).

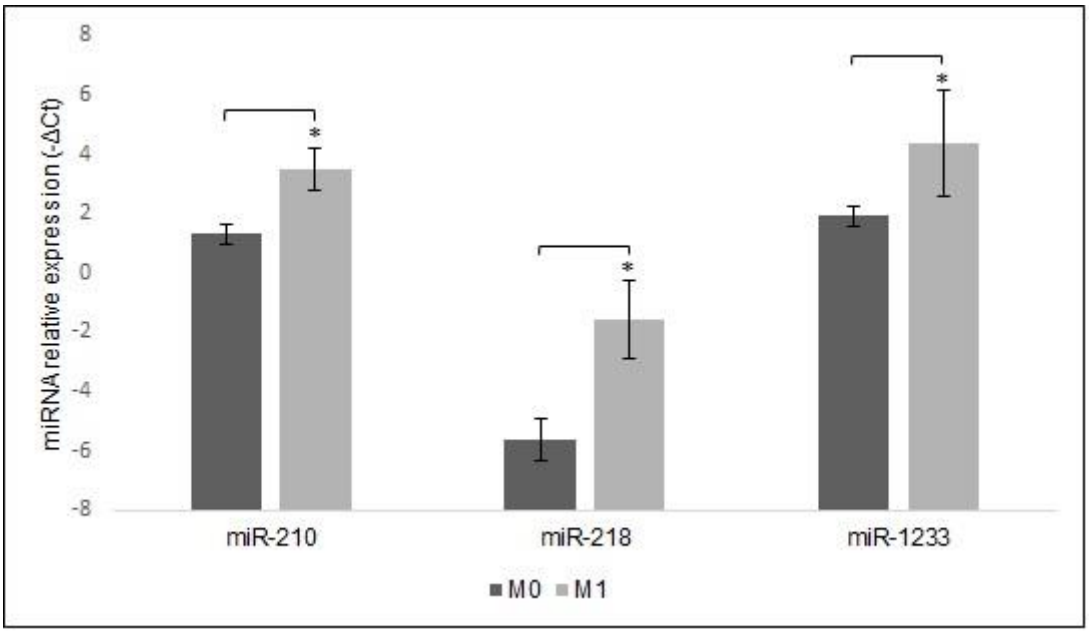


Figure 19 - Relative expression of miR-210, miR-218 and miR-1233 considering the presence of metastatic disease (Mean ± Std.Error; *$P \leq 0.050$).

Attending the dispersion of the circulating expression levels of miR-210 and miR-1233, we defined three miRNA expression groups among the RCC patients. Regarding the synergic expression of miR-210 and miR-1233, we observed a lower overall survival in patients who belonged to group 1 (higher expression levels of both miR-210 and miR-1233) compared to group 2 and 3 (intermediate and lower levels of both miR-210 and miR-1233, respectively). Since we only observe an increase of miR-210 and miR-1233 when considering microvascular invasion, tumor size and metastasis at the moment of diagnosis, we analyzed the synergic influence of both miRNAs in the overall survival of RCC patients (Figure 20). We observed that RCC patients that presented higher circulating levels of both miR-210 and miR-1233 had a lower overall survival when compared with patients with lower expression of these two miRNAs (Log Rank test *P* = 0,036).

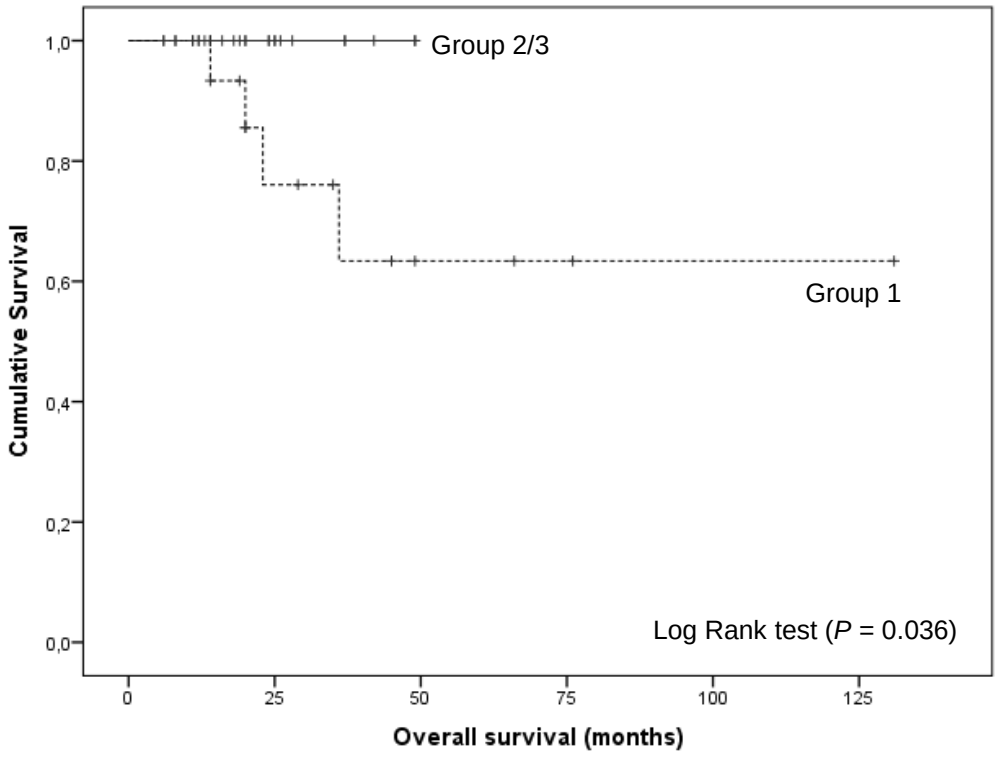
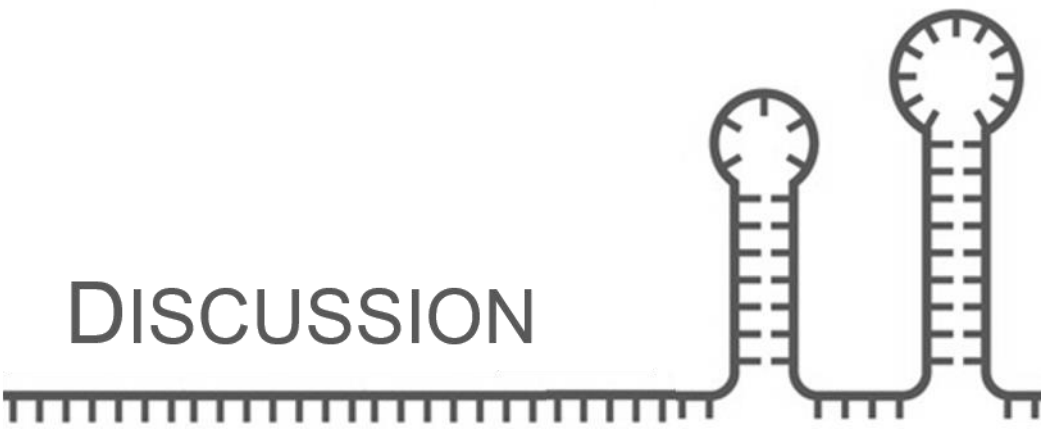


Figure 20 - Overall survival by Kaplan-Meier and Log Rank test (*P* = 0.036) of RCC patients, according to the expression of miR-210 and miR-1233 in the plasma of RCC patients.

DISCUSSION



5. Discussion

Hypoxia is a unique environmental stress that induces global changes in a complex regulatory network of transcription factors and signaling pathways in order to coordinate cellular adaptations in metabolism, proliferation, DNA repair, and apoptosis [102]. As previously described, one of the early molecular events in the oncobiology o RCC is the loss of *VHL* gene which leads to an increase of HIF-α and, consequently, triggers and hypoxic response from the cell [46, 49]. Several lines of evidence now establish miRNAs as key elements in this cellular response to hypoxia [102-104].

HIF-responsive miR-210 is a unique miRNA that is evolutionary conserved and ubiquitously expressed in hypoxic cell and tissue types [105]. This miRNA is located in the intronic sequence that codifies *AK123486* mRNA transcript that is hypoxia inducible, and is also flanked by, *HRAS* and *RASSF7* genes which are also regulated by hypoxia [106, 107]. These characteristics make this miRNA an accurate indicator of HIF-α pathway activation. MiR-210 has multiple direct targets and exerts its influence on a wide range of cellular responses known to influence normal developmental physiology, such as: proliferation, differentiation, mitochondrial metabolism, protein modification, nucleic acid binding, migration and angiogenesis (Figure 21) [108].

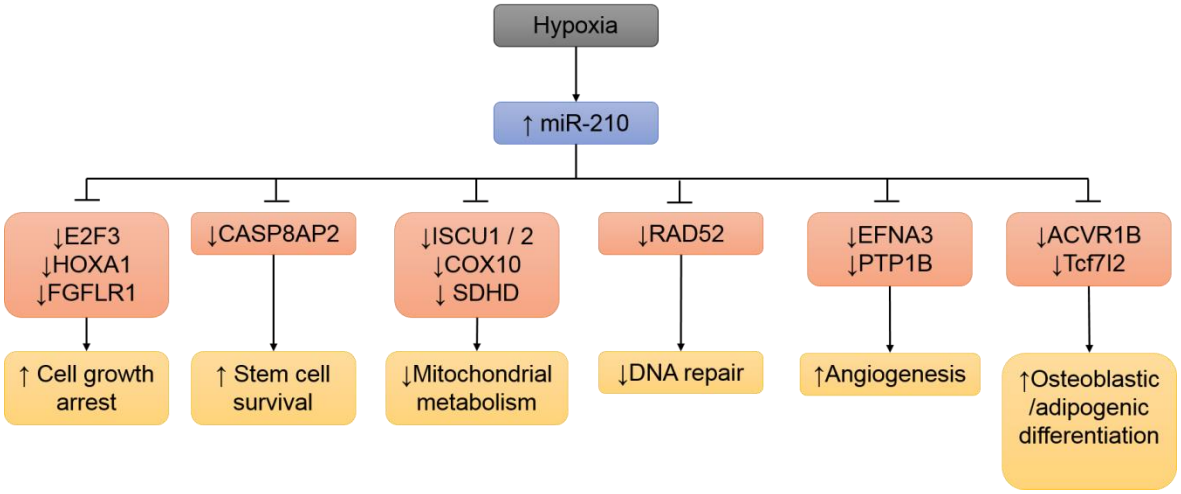


Figure 21 - Summary of miR-210 targets and their biological consequences under hypoxia. (E2F3 - E2F Transcription Factor 3; HOXA1 - Homeobox A1; FGFLR1 - Fibroblast Growth Factor 1; CASP8AP2 - Caspase Associated Protein 2; ISCU1/2 - Iron-sulfur Cluster Assembly Enzyme 1/2; COX10 - Cytochrome C Oxydase 10; SDHD - Succinate Dehydrogenase Complex Subunit D: EFNA3 - Ephrin-A3; PTP1B - Protein Tyrosine Phosphatase, non-receptor type 1; ACVR1B - activin A receptor, type IB; Tcf7l2 - Transcription Factor 7-like 2) (Adapted from Chan *et al.* 2012 [105]).

Among miR-210 targets, EFNA3 and PTP1B are involved in angiogenesis and CASP8AP2 is involved in stem cell survival, which make this miRNA a potential biomarker for prognosis and aggressiveness [108]. Recently, studies performed by Zhao and coworkers showed that miR-210 was overexpressed in serum samples of patients with RCC compared to healthy controls, which is consistent with the results obtained in the present study [66]. They also described that the average serum level of miR-210 was significantly decreased in patients 1 week after renal resection surgery, suggesting that this miRNA is released from the tumor tissue into the bloodstream [66]. This result is also consistent with what we obtained since we observed an expression 7.84 times higher of miR-210 in plasma of patients with larger tumors, which supports the idea that indeed miR-210 could be secreted from the tumor. Valera and colleagues also observed an overexpression of miR-210 in tumor tissue from RCC patients and were able to associate increased expression levels of miR-210 with higher Fuhrman nuclear grade tumors and with lymph node metastasis [109]. In the present study we didn't observe any relation with the plasma expression levels of miR-210 and Fuhrman nuclear grade. However, we observed a 4.63-fold increase in plasma expression levels of miR-210 in patients that presented metastasis at diagnosis. It is important to note that the present study was conducted in plasma samples while Valera and coworkers study was conducted in tumor tissue samples, which can explain our results for Fuhrman nuclear grade.

Hypoxia stimulates the increase of numerous miRNAs, but at the same time it's responsible for the downregulation of others, such as miR-218 [110]. Mathew and coworkers demonstrated that elevated levels of HIF-α were related to low levels of miR-218 and that low levels of miR-218 increased the expression of multiple components of the EGFR pathway [110]. Moreover, results of previous functional studies of miR-218 in various cancers indicate that this miRNA inhibits cancer cell proliferation and invasion. These results strongly indicate that it functions as tumor suppressor miRNA [111-114]. Regarding RCC, Yamasaki and coworkers observed that miR-218 inhibits cell migration and invasion through targeting *Caveolin-2* and that this miRNA is downregulated in RCC. They came to this conclusion by performing *in vitro* studies using cell lines and also using RCC tumor tissue samples [98]. However, the results obtained in the present study aren't in agreement with the literature since we observed (both *in vitro* and *in vivo*) an increase in the extracellular expression levels of miR-218. Once again it is important to note that the majority of studies regarding miR-218 expression are performed using tumor tissue samples and in the present study we observe an increase of miR-218 in the cells medium and in the plasma of RCC patients but we don't analyze its expression in tissue samples. Regarding the explanation to the results obtained we hypothesize that, since miR-218 is known as a tumor suppressor miRNA that targets EGFR and that this pathway is

deregulated in RCC, the excretion of miR-218 from the tumor cells could be an autocrine attempt from the cell to compensate EGFR pathway deregulation. Furthermore, miR-218 also has de ability to inhibit the anti-apoptotic molecule Survivin [98]. Higher levels of Survivin are associated with higher RCC grade and poor prognosis [115-117]. Taking this into account, we can also hypothesize that the increase of the expression levels of miR-218 function as a resistant mechanism from the cell in order to stop RCC progression. However, further studies are needed to validate these hypotheses.

Recently, the miR-1233 has been described by Wulfken and colleagues as a potential biomarker in RCC, since an increase in its serum levels was detected in RCC patients when compared to healthy controls [62]. This result is consistent with we obtained in the present study and, to the best of our knowledge, we are the first group to relate the extracellular expression levels of miR-1233 with clinicalpatholocial characteristics from RCC patients. Our results indicate that, besides being overexpressed in the plasma of RCC patients, miR-1233 extracellular are also higher in patients with larger tumors, higher Fuhrman nuclear grade, higher microvascular invasion and presence of metastasis, which reinforces the potential of this miRNA in becoming a biomarker in RCC. Regarding its function or what even induces its transcription, there is not much information about miR-1233, but two of its potential targets are *BLCAP* (identified as a tumor suppressor gene in bladder cancer) and *p53* [62].

Recent evidence indicates that HIF alone is insufficient to implement the complete program of adaptative changes required for cell survival under hypoxic stress [102]. The p53 protein is a transcription factor, which is rapidly induced by cellular responses to hypoxia and can also work together with HIF to regulate the hypoxic response from the cell [118-120]. According to a model proposed by Sermeus and colleagues, under basal normoxic conditions, the low abundance of p53 and HIF-1α induces the expression of targets genes that are involved in survival. The early state of hypoxia increases the HIF and reduces the p53 in order to activate the hypoxic response and avoid the activation of pro-apoptotic signals, respectively. But if the oxygen concentration continue to decrease or hypoxia lasts too long, HIF-1α induces p53 increase and stabilization, leading to the activation of pro-apoptotic genes and, consequently, to apoptosis [118].

Moreover, hypoxia is a primary event in RCC, due to the loss of VHL, and is as well-established inducer of angiogenesis and vascularization necessary for further tumor development. Under these conditions HIF-α expression leads to the transcription of target genes involved in growth of blood vessels such as VEGF, inducible nitric oxide synthase, fibroblast growth factor and matrix metalloproteinases [46, 49]. In response to this, p53 is able to limit tumor vascularization in three different ways: by increasing the production of antiangiogenic factors; directly inhibit HIF-α pathway by direct competition for HIF-α p300

co-factor and finally through the repression of genes encoding pro-angiogenic factors, including basic fibroblast growth factor and VEGF [121, 122]

So, based on the previous description of p53 functions under hypoxic conditions, we can hypothesize that, through targeting and inhibition of p53, miR-1233 is able to promote a continuous state of hypoxia, cell survival and angiogenesis since it stops p53 from doing its function.

Considering that both miR-210 is induced by hypoxia and miR-1233 helps maintaining the hypoxia state, and we observed higher circulating values of both these miRNA in RCC patients with larger tumors, microvascular invasion and metastasis, we hypothesized that these two miRNAs could be potential biomarkers for aggressiveness and prognosis in RCC. In fact, when we performed an overall survival analyzes considering the expression of both miR-210 and miR-1233 we observed that the patients that present simultaneous higher expression of both these miRNAs died early, which sustains our hypothesis.

Thus, we propose that these patients, with high expression levels of both miR-210 and miR-1233, will present a higher stimulus to cell growth, angiogenesis, stem cell survival and apoptosis inhibition, which translates in a poor overall survival (Figure 22).

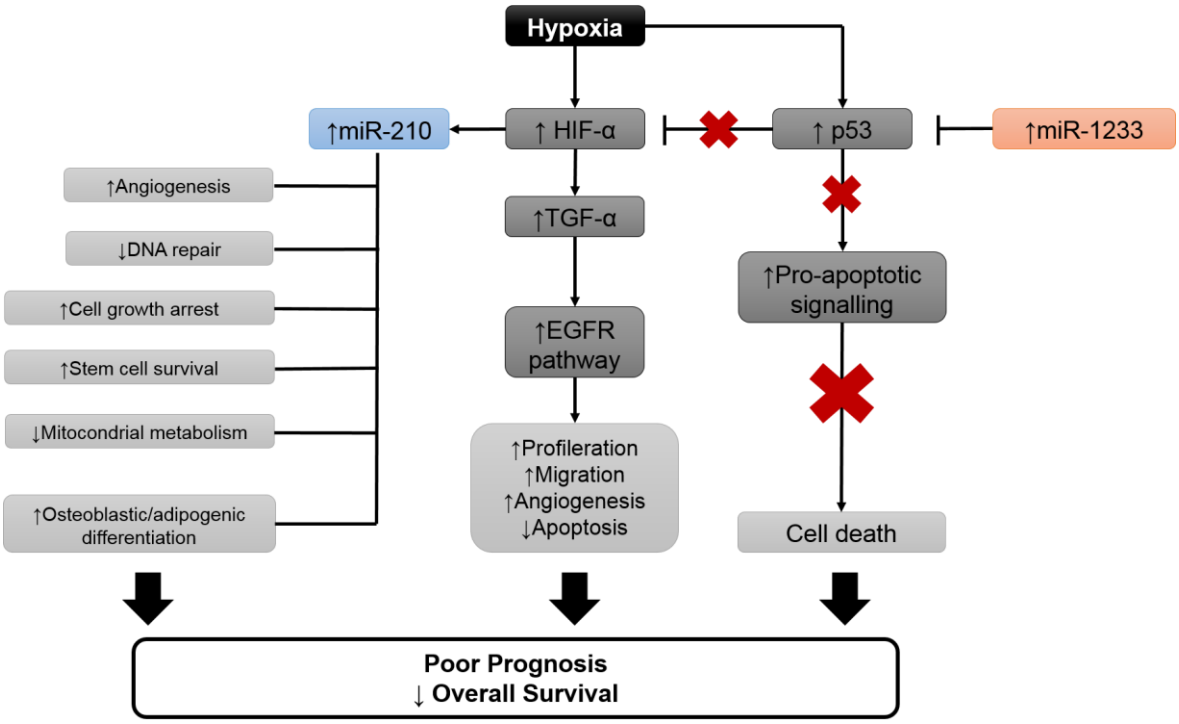
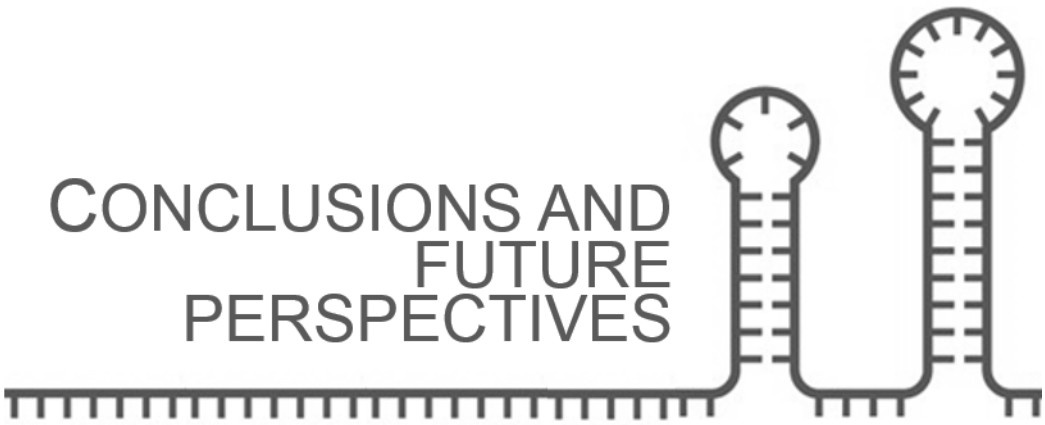


Figure 22 - Proposed model of miR-210 and miR-1233 role during RCC development, according to the results obtained in the present study.

CONCLUSIONS AND FUTURE PERSPECTIVES



6. Conclusions and Future Perspectives

The reality of the non-existence of a standard screening test for the early detection and follow-up in RCC has been haunting the field of Oncology for the past years once RCC is the most lethal urologic neoplasia.

The hypothesis of exosome-dependent signaling between cells has been revolutionizing molecular biology and giving space for reformulating the idea of tumor microenvironment and the mechanism by which metastization occurs. Exosomes are small microvesicles that present high stability in several body fluids and can be released from a wide range of cell, including cancer cells. They can be uptaken by neighboring cells and are capable of inducing pathways involved in cancer initiation and progression.

Among the cargo that can be transported through exosomes we can find miRNAs. MiRNAs are small non-coding RNAs responsible for the regulation of numerous genes at a post-transcriptional level. In the last few years they have been widely studied in cancer since they can act as oncomiRNAs (if they inhibit tumor suppressor genes) or tumor suppressor miRNAs (if they inhibit oncogenes).

Recently, expression profiling has emerged as a powerful approach to identify the molecular mechanism underlying carcinogenesis and to predict clinical outcomes, helping to identify new biomarkers of aggressiveness and prognosis. The analysis of miRNAs expression patterns offers an opportunity for the identification of several tumors, since some of these molecules appear to be differentially expressed in normal *versus* cancer tissues and in different phases of cancer development.

In the present study we found that miR-210 and miR-1233 (classified as oncomiRNAs) presented high expression levels in the plasma samples of RCC patients and that those higher levels are associated to higher tumor size, higher Fuhrman nuclear grade, higher microvascular invasion and metastasis at the time of diagnosis, which made them eligible for potential biomarkers of prognostic and aggressiveness in RCC. To support our hypothesis, we observed that patients with higher expression levels of these miRNAs in plasma presented a poor overall survival.

However, there are some limitations in this study. We only used circulating samples from RCC patients in order to study potential non-invasive biomarkers but in the future it would be useful to study the corresponding tumor samples in order to study the relationship between the intercellular/extracellular levels of these miRNAs, as we did for the *in vitro* phase of the study. Future studies must include a higher number of miRNAs. We started with a panel of five miRNAs, but only three passed the *in vitro* phase of the study and in the end only two were related with RCC patients overall survival. Thus, as

future perspectives it would be interesting to: replicate this study in a larger number of RCC patients to validate the results obtained in this study; collect plasma samples before and after surgery in order to validate the hypothesis that the miRNAs are released from the tumor to the bloodstream; collect tumor samples to compare with the plasma samples and search for more deregulated miRNAs in RCC in order to improve the established miRNA profile.

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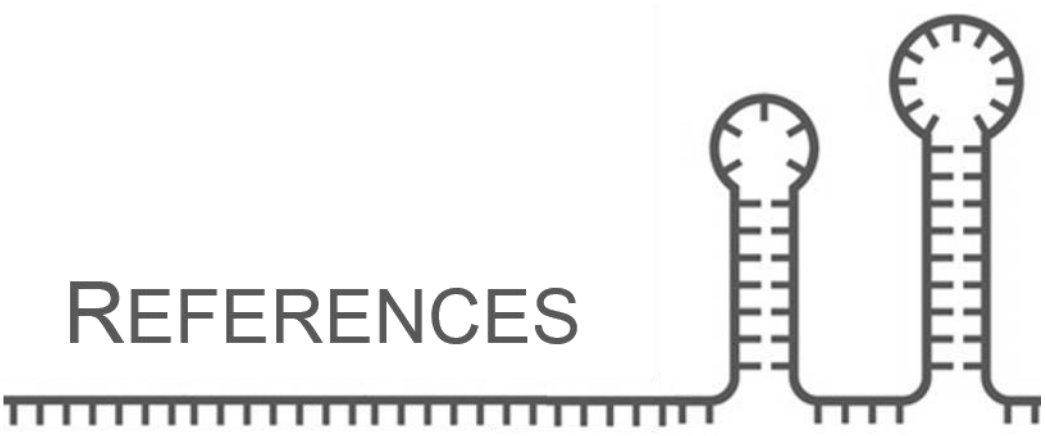

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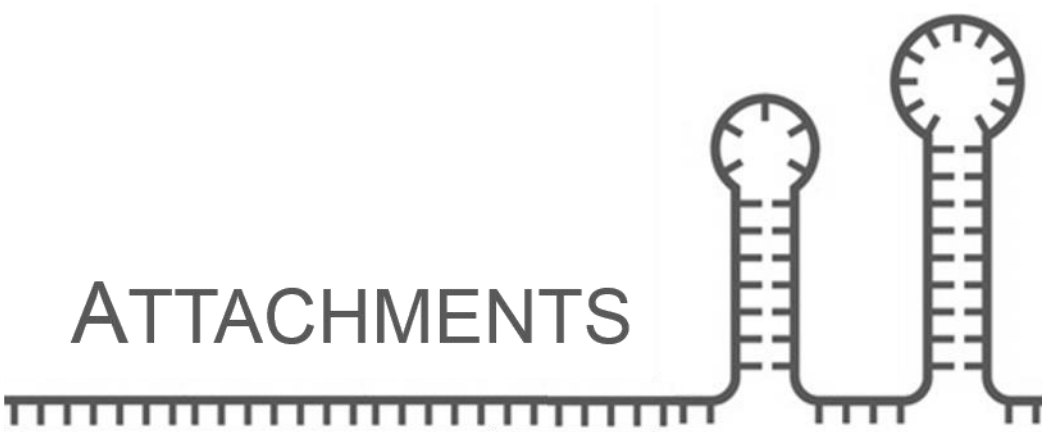
ATTACHMENTS



Renal cell carcinoma development and miRNAs: a possible link to the EGFR pathway

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney and the majority of RCC cases are detected accidentally. This reality and the nonexistence of a standard screening test contribute to the fact that one third of patients are diagnosed with local invasive disease or metastatic disease. miRNAs are a family of small ncRNAs that regulate gene expression and have been identified as key regulators in many biological processes including cell development, differentiation, apoptosis and proliferation. The EGF receptor signaling pathway is usually deregulated in cancer and it is suggested to have an important role in RCC. Further studies are needed to characterize deregulation of this pathway during RCC development. In this review we highlight some potential miRNAs that could be involved in the modulation of the EGF receptor pathway and consequently in RCC development.

KEYWORDS: biomarkers ■ EGFR ■ miRNAs ■ renal cell carcinoma ■ targeted therapies ■ VHL

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Epidemiology of renal cell carcinoma

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney, accounting for approximately 90% of kidney neoplasms and 3% of all adult malignancies [1,2]. According to the European Association of Urology, there is a 1.5:1 predominance of new cases diagnosed in men over women, with a peak of incidence occurring between the age of 60 and 70 years [2]. Worldwide mortality, as a result of RCC, currently exceeds 100,000 patients each year, with the incidence and mortality rate increasing by 2–3% per decade [3]. According to the 2004 WHO classification, the three most prevalent kidney cancer histological subtypes are clear cell RCC, papillary RCC and chromophobe RCC (with a prevalence of 70, 10 and 5%, respectively) [4].

Actually, the majority of RCC cases are detected accidentally as a consequence of diagnostic tests (computed tomography, MRI and so on) owing to symptoms that are not specifically related to the disease [1,5]. This reality and the nonexistence of a standard screening test, contributes to the fact that one-third of patients are diagnosed with local invasive disease or metastatic disease [6]. Although surgical resection remains the best curative therapy approach for RCC, 20–40% of patients will develop recurring disease after surgical nephrectomy [7], due to the inexistence of adjuvant therapy in clinical routine [4]. RCC is both chemotherapy and radiotherapy resistant [8]. Another issue related to this type of cancer is the absence of biomarkers for the early detection and follow-up of the disease, which complicate the early diagnosis and makes it a challenge for the field of oncology [8].

Actually, despite intensive efforts, the mechanisms involved in RCC development and progression remain under study. One of the signaling pathways involved in the pathophysiology of RCC is the von Hippel–Lindau (VHL) pathway [9–13]. In normoxic conditions, the protein encoded by the *VHL* gene serves as a recognition site for the regulatory subunits of HIF, targeting them for proteosome degradation (Figure 1A). One of the early molecular events of RCC is the loss of *pVHL* (a consequence of the loss of the short arm of chromosome 3), which stops the degradation of HIF and leads to its accumulation in the cytoplasm and further migration to the nucleus where it binds to hypoxia-related genes, leading to a hypoxic response from the cell. Once activated, these genes are involved in pathways responsible for the development of blood vessels, proliferation, glucose metabolism, pH regulation and metastatic disease (Figure 1B) [14–16]. One of the activated genes is TGF-α, which is involved in the induction of cellular proliferation by activating the EGF receptor (EGFR) [17]. This receptor is a membrane glycoprotein that belongs to the ErbB family of tyrosine kinase receptors [18]. The EGFR activates several signaling pathways, including the MAPK/ERK and PI3K/AKT pathways [19]. These pathways can modulate genetic transcription, stimulating cellular proliferation, migration, invasion, angiogenesis and apoptosis inhibition (Figure 1C) [20].

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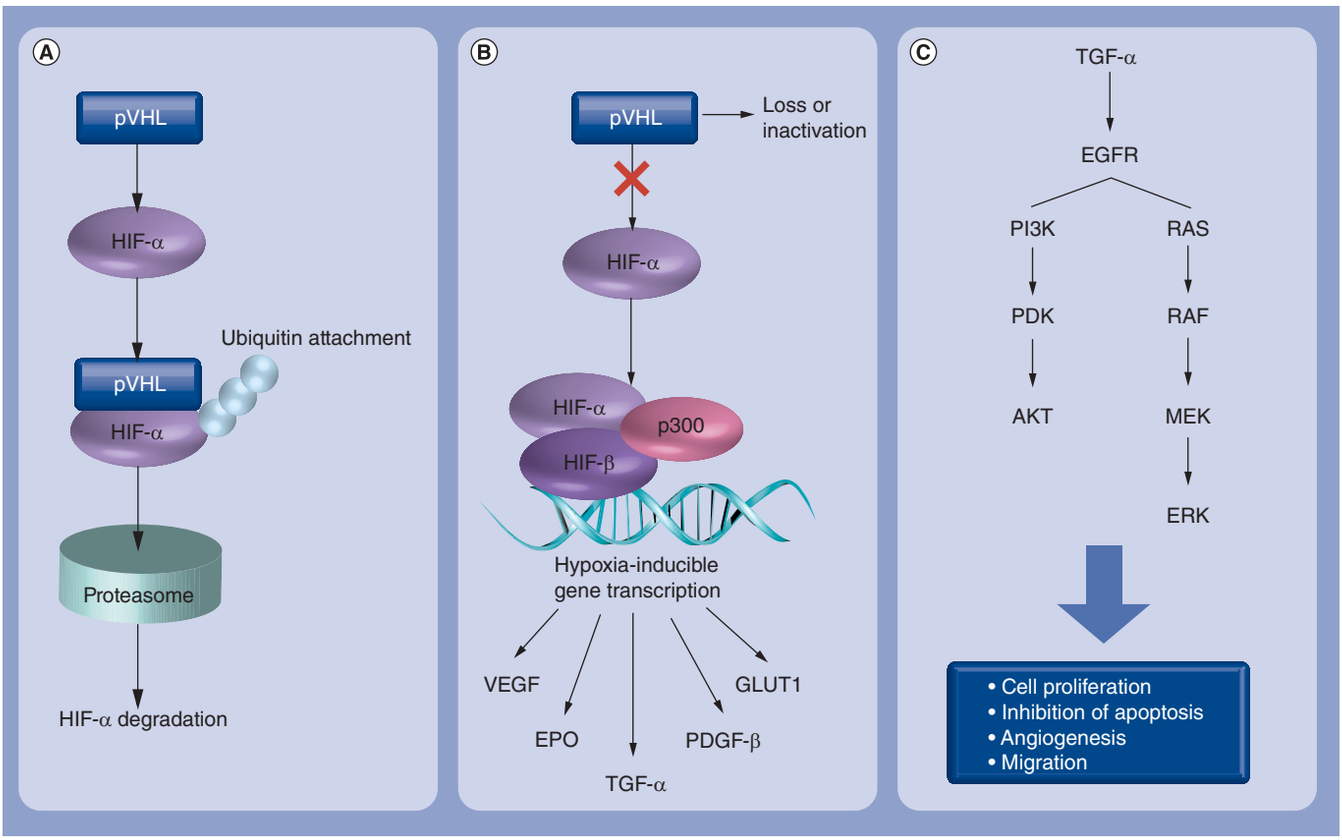

Figure 1. Signaling pathways involved in renal cell carcinoma pathophysiology. (A) pVHL pathway under normoxic conditions: pVHL targets HIF-α for degradation in the proteosome. **(B)** pVHL pathway under hypoxic conditions: the loss of pVHL leads to the accumulation of HIF-α in the nucleus and consequent binding to transcription factors, which triggers a hypoxic response from the cell. **(C)** EGFR pathway activation by TGF-α and its functional consequences.

EGFR overexpression is found in approximately 30% of primary tumors and has been associated with advanced disease, poor prognosis, overall survival and therapy response [21,22]. The VHL is also responsible for EGFR turnover and further degradation in the proteosome, thus during the RCC development these processes could be compromised, leading to an increase of the EGFR [23]. Zhou and coworkers described EGFR half-life as approximately 1 h in 786-VHL cells but approximately 3 h in 786-mock cells and that both the phospho-AKT and the phospho-ERK signals lasted longer in 786-mock cells than in 786-VHL cells when stimulated with EGF [23]. If we relate the increase in EGFR half-life with the increase of *TGF*-α production, it becomes clear the relevance of the study of this pathway in RCC. It has also been described that hypoxia upregulates EGFR and prolongs its activation through its retention in endocytic trafficking [24]. Recently, Shen and colleagues demonstrated that EGFR is upregulated during tumor progression and specifically enriched in hypoxic tumor areas. They also described EGFR suppression of miRNA maturation through AGO2-Y393 phosphorylation under hypoxic conditions [25].

In recent years, the increasing knowledge of the pathways involved in RCC has allowed for the development of new targeted therapies. The identification of the influence of the *VHL* gene in RCC led to the development of targeted therapies such as sunitinib and sorafenib (tyrosine kinase domain inhibitors), as well as pazopanib (angiogenisis inhibitor); these therapies are directed towards this molecular pathway [15,26]. However a subset of patients (~25%) do not seem to experience any clinical benefit from targeted therapy, while in the majority of cases, patients respond to therapy initially but go on to experience disease progression [27]. Usually resistance to the targeted agents has been shown to develop after a median of 5–11 months of treatment and a small subset of patients do not experience any clinical benefit from the targeted therapy [28]. These 'failures' of targeted therapies show us the necessity of further investigation into the molecular pathways involved in RCC in order to improve our understanding about molecular events in this

type of cancer and to allow for the development of new effective targets and therapies.

miRNAs & cancer

miRNAs (also known as miRs) are a family of small ncRNAs (19–25 nucleotides in length) that regulate gene expression by sequence-selective targeting of mRNAs, leading to degradation or blockade of mRNA at the post-transcriptional level, depending on the degree of complementarity between miRNAs and the target mRNA sequence [29,30].

For the majority of miRNAs, the primary transcripts are generated in the nucleus [31]. Following transcription, the pri-miRNA is processed by DROSHA and its binding partner DGCR8 (also known as Pasha), creating a pre-miRNA [32]. The pre-miRNAs are exported to the cytoplasm by the nuclear export protein XPO5, where they are further processed by DICER, leading to the production of mature approximately 22 nucleotide double-stranded molecules [33]. The mature miRNA enters the miRISC complex where it is further processed, leaving a single-stranded functional miRNA. This single-stranded miRNA then induces post-transcriptional gene silencing by guiding the miRISC complex to target mRNAs. Target recognition occurs mainly by incomplete base pairing complementarity between the miRNA and the target mRNA, resulting in mismatches that, in turn, lead to target gene silencing, which can occur via translational repression and/or mRNA degradation (FIGURE 2) [33,34].

miRNA expression is dynamic, so may be altered within the same cell. This variability makes miRNAs potent modulators of cellular behavior, since several miRNAs can target the same gene and one single miRNA can target multiple genes [35]. Hence, miRNAs have been identified as key regulators in many biological processes including cell development,

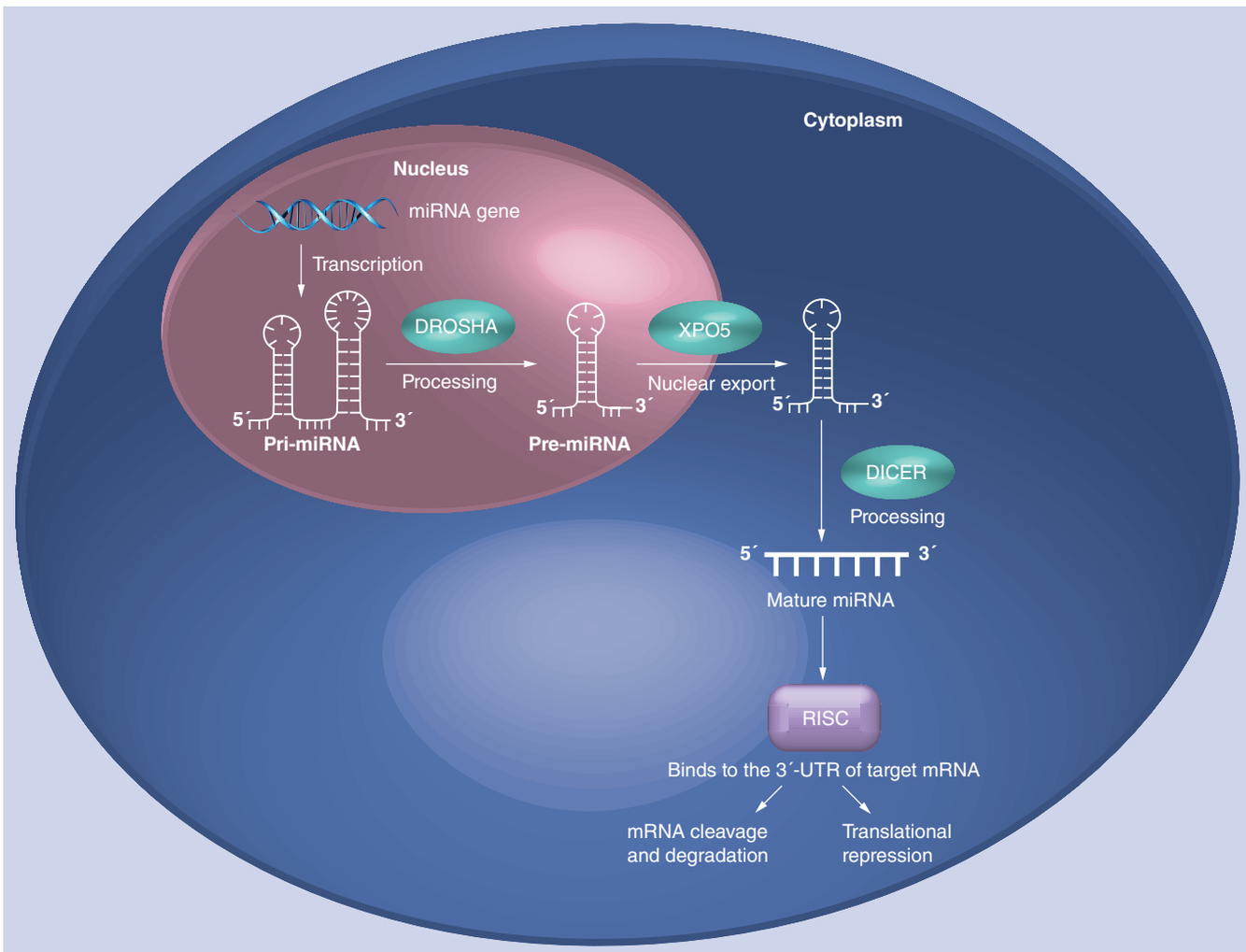

Figure 2. miRNA biogenesis and processing mechanism.

differentiation, apoptosis and proliferation [36]. miRNAs deregulation can modulate the renal cell microenvironment, which can impact on cancer development. We previously observed that slight differences in microenvironment, owing to functional SNPs, can be associated with RCC development [37].

As a single miRNA may target up to several hundred mRNAs, aberrant miRNA expression may affect a multitude of transcripts and profoundly influence cancer-related signaling pathways [31]. Many miRNAs have been identified to act as oncogenes, tumor suppressors or even modulators of cancer stem cells and metastasis formation [38]. OncomiRs are known to downregulate tumor suppressor genes, and have been reported to be overexpressed in multiple miRNA expression-profiling studies. On the other hand, tumor suppressor miRNAs are responsible for downregulating oncogenes, and are mostly underexpressed in cancer [33]. Duns and coworkers reported that the miR-200 family, a family of tumor suppressor miRNAs known to be implicated in the epithelial-to-mesenchymal transition process, are deregulated in clear cell (ccRCC) [39].

miRNAs represent attractive drug targets since they regulate the expression of several cellular proteins and are differentially expressed in malignant cells when compared to normal cells.

miRNA expression modulation during RCC development

The EGFR–PI3K–AKT pathway is frequently overexpressed in ccRCC [40]. In this section we will focus in some miRNAs that have been described as frequently deregulated in RCC and relate them to the EGFR pathway. Since the majority of deregulated miRNAs involved in this pathway are upregulated in RCC, we will focus on these miRNAs. To the best of our knowledge no reports have been published relating downregulated miRNAs in RCC to the EGFR pathway.

miR-21 is described in the literature as presenting an oncogene role in cancer development [41–43]. In a variety of cancer cell lines, overexpression of miR-21 could increase cell proliferation, migration, invasion and survival, while its suppression could induce apoptosis and repress cell proliferation and invasion capacity (Table 1) [44]. In a large-scale meta-analysis of miRNA expression using 540 human samples of six types of solid tumors, miR-21 was the only miRNA upregulated in all the tumors, therefore overexpression of miR-21 may be a general feature of carcinoma and be used as a biomarker [44]. miR-21 transcription can be induced by AP1, NF-κB and RAS/ERK signaling pathways and this miRNA has multiple targets involved in different cellular processes [45]. Some of the targets of miR-21 include p53 and Cdc25A, which are related to cell cycle control; TIMP1, TIMP3 and RECK, which are related to invasion; and PTEN, PDCD4 and FasL, which modulate apoptosis [45]. Regarding RCC, Zhang and colleagues demonstrated that aberrantly expressed miR-21 regulates the TCF21–KISS1-associated RCC invasion pathway, and this miRNA signature could offer a novel potential therapeutic strategy for RCC [41].

Recently, Liu and coworkers demonstrated that miR-21 could induce tumor angiogenesis through targeting of PTEN, leading to the

Table 1. Chromosome location, predicted target genes and functional consequences of miR-21, miR-155, miR-210, miR-221, miR-222 and miR-1233 in the context of renal cell carcinoma and EGFR pathway.

miRNA	Location	Predicted genes	Consequence	Ref.
miR-21	17q23.1	*p53, Cdc25A, TIMP1, TIMP3, RECK, PTEN, PDC4* and *FasL*	↑ proliferation ↑ invasion ↓ apoptosis	[45,46]
miR-155	21q21.3	*HIF-1α*	↑ proliferation ↓ apoptosis	[48]
miR-210	11p15.5	*EFNA3, PTP1B* and *CASP8AP2*	↑ stem cell survival ↑ invasion	[52]
miR-221/222	Xp11.3	*PTEN, TIMP3* and *TRPS1*	↑ invasion ↑ proliferation ↓ apoptosis	[19,54–56]
miR-1233	15q14	*BLCAP* and *p53*	↑ proliferation	[58]

↑: Increase; ↓: Decrease.
Data taken from [101].

activation of AKT and ERK1/2 signaling pathways, and thereby enhancing HIF-1α and VEGF expression [46]. Cells treated with an antisense-miR-21 showed a decreased expression of EGFR, activated AKT, cyclin D and Bcl-2 leading to cell proliferation and differentiation inhibition [47].

Another miRNA involved in hypoxia response is miR-155. This miRNA is induced by hypoxia and plays a role as a component of a network of negative feedback loops that controls *HIF-1α* translation [48]. It has also been reported that miR-155 is involved in the downregulation of many tumor suppressor genes that repress PI3K related pathways [49]. As a consequence, pathways involving EGFR will be constantly activated. miR-155 is significantly upregulated in ccRCC compared with normal cells and high expression levels of miR-155 were correlated with increased tumor size [49,50]. On the other hand Shinmei and coworkers also described a suppression of miR-155 expression in patients with stage III and stage IV ccRCC and correlated it with a poor prognosis [49]. These data suggest that miR-155 may have different roles during ccRCC progression: initially its overexpression is related to an increase in tumor size and, when the tumor reaches stages III and IV, a suppression of miR-155 occurs and is related to a poorer prognosis for the patient. Although more studies are needed to explain this transition, this phenomenon of over- and under-expression of miR-155 may be explained by its involvement in the network of negative feedback loops that control *HIF-1α* translation.

miR-210, is another miRNA that is regulated by HIF-1α. HIF-1α accumulation in the cell is a key event in RCC owing to *VHL* mutation, therefore we expect to find higher levels of this miRNA in RCC patients [51]. As explained earlier, HIF-1α accumulation also results in the transcription of TGF-α, which in turn will stimulate the EGFR pathway. So, we can expect that an overexpression of miR-210 and TGF-α could be related since they are both regulated by HIF-1α (FIGURE 3). Among miR-210 targets, EFNA3 and PTP1B are involved in angiogenesis and CASP8AP2 is involved in stem cell survival [52]. Recently, studies performed by Zhao and coworkers showed that miR-210 was overexpressed in serum samples (n = 68) of patients with RCC compared with healthy controls (n = 32; with an area under the curve of 0.874, a sensitivity of 81% and a specificity of 79.4%) [53]. They also described that the average serum level of miR-210 was significantly decreased

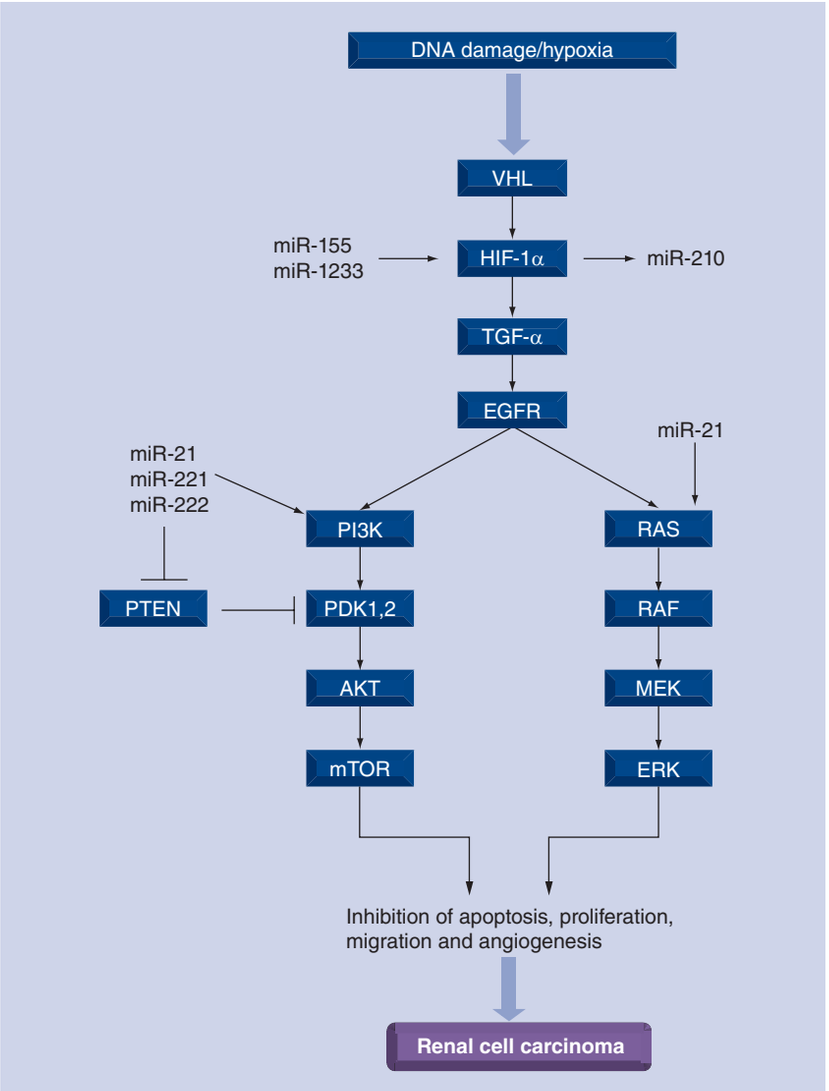

Figure 3. Link between the VHL and EGFR pathways, the miRNAs involved and their target genes.

in patients 1 week after resection surgery ($p < 0.001$), suggesting that this miR is released from the tumor tissue into the bloodstream [53]. According to a report by Valera and coworkers, an overexpression of miR-210 in RCC patients was observed and increased expression levels of miR-210 are associated with higher Fuhrman nuclear grade tumors and tumors with lymph node metastasis [51].

During RCC development, TGF-α transcriptional activation may result in cellular proliferation induction as a consequence of PI3K/AKT pathway activation. The PI3K/AKT pathway is correlated with apoptosis resistance, increased proliferation, cellular migration and survival signaling (normally dependent on growth factors) [54]. This pathway activation can lead to an increase in miR-221 and miR-222 expression

levels. These two miRNAs can target the 3′-UTR sequence of *PTEN* and *TIMP3* leading to their degradation [54,55]. The upregulation of miR-221/222 leads to the downregulation of *PTEN* and consequent activation of AKT (Figure 3) [19]. Works performed by Zhang and coworkers described that the activation of AKT by the downregulation of *PTEN* is related to the gain of radioresistance and when the two miRNAs are knocked down, the expression of *PTEN* is re-established, resulting in an enhancement in radiation-induced apoptosis in tumor cells [54]. miR-221/222 are also involved in the metastatic process. Their overexpression is related to cellular invasion by directly targeting TIMP3, an inhibitor of metalloproteinases, and by repressing transcriptional factors, such as the TRPS1 [54,56]. The repression of this factor causes an increase in the levels of the ZEB2, which promotes a crucial step in the epithelial-to-mesenchymal transition, essential for the development of metastasis [56,57]. Recently, the miR-1233 has been described by Wulfken and colleagues as a potential biomarker in RCC, since an increase in its serum levels was detected in RCC patients (n = 30) when compared with healthy controls (n = 33; sensitivity 77%, specificity 37.6%, area under the curve 0.588) [58]. Regarding its function, there is not much information about miR-1233, but two of its potential targets are *BLCAP* (identified as a tumor suppressor gene in bladder cancer) and *p53*, so we can hypothesize that it is related to the regulation of tumor suppressor genes [58].

Since p53 can target and inhibit HIF-1α, it is possible that miR-1233 overexpression results in the inhibition or suppression of p53 which in turn will result in HIF-1α activation [59,60]. This will lead to TGF-α transcription and EGFR pathway activation (Figure 3).

miRNAS as potential biomarkers for diagnosis & prognosis in RCC

Owing to RCC characteristics, new and precise biomarkers are needed for early detection and for follow-up regarding disease progression. The characterization of these new biomarkers could be an interesting approach for the identification of novel therapeutic targets and could accelerate the development of new drugs for RCC treatment.

miRNAs as biomarkers in body fluids

Besides the fact that miRNAs are differentially expressed in normal cells versus cancer cells, miRNA expression signatures in the blood, serum and plasma are similar in men and women, as well in individuals of different ages [58]. On the other hand, levels of miRNAs in circulating samples are reproducible and consistent among individuals of the same species and specific expression patterns of serum miRNAs have already been identified for pregnancy, diabetes and different cancers, thus providing evidence that serum miRNAs contain fingerprints distinctive of certain human conditions [19]. These characteristics make them good candidates for cancer-specific biomarkers.

Regarding RCC, some miRNAs have been suggested as serum biomarkers: miR-378, as it is known to promote cell survival and angiogenisis and is upregulated in the serum from patients with RCC; whereas miR-145, which sensitizes cells to glucose deprivation, is downregulated in serum from patients with RCC [61]. Recently, our group observed that miR-222 expression levels are increased in RCC patients compared with healthy individuals, and the higher expression levels of miR-222 are also associated with a lower overall survival [62] [Teixeira AL, Gomes M, Medeiros R. Circulating miR-221/222 in renal cell carcinoma: a new potential biomarkers for diagnosis and prognosis in Portuguese population (2012), Submitted]. Despite the concept of using miRNAs for cancer diagnosis and prognosis seeming very promising, RCC-specific biomarkers have not yet been identified and more studies are needed to establish a solid miRNA signature for RCC diagnosis [63].

miRNAs as biomarkers in tissue samples

Another example of the utility of miRNAs as potential biomarkers for diagnosis is a study performed by Jung and coworkers in which they identified a robust signature to define ccRCC, with five significantly overexpressed and six significantly downregulated miRNAs in cancer tissue samples. A combination of upregulated miR-155 and downregulated miR-141 is found to result in a 97% overall correct classification of the matched malignant and nonmalignant tissue samples [64]. In stage II colorectal cancer miRNA expression profiles were capable of predicting recurrence rates with an accuracy of >80%, suggesting that miRNA profiling can also be used to determine a tumor's aggressiveness [65].

In addition, miRNA expression patterns can help distinguish tumor histopathological subtypes. Youssef and coworkers developed a classification system, using microarray analysis that can distinguish the different RCC subtypes using unique miRNA signatures in a maximum of four

steps. The system has a sensitivity of 97% in distinguishing normal RCC, 100% for clear cell RCC subtype, 97% for papillary RCC subtype, and 100% accuracy in distinguishing oncocytoma from chromophobe RCC subtype [66].

Taken together, these data suggest that circulating miRNAs have the potential to become powerful and sensitive biomarkers, which can be easily detectable in noninvasive assays that might overtake the antibody-related drawbacks of proteomics approaches and could help in the diagnosis and prognosis of several types of cancer, namely RCC.

miRNAs as potential tools for targeted therapy

In general, technological advances are enabling the synthesis of pre- or anti-RNA molecules within carrier vehicles that can be administered topically or systematically to induce generalized cell targeting. If the targeted event is cancer specific, then the effects should be harmless to normal cells and antineoplastic cells (FIGURE 4) [35]. There are four types of miRNA delivery strategies to restore miRNA levels in cancer: antagomirs and locked nucleic acids (which do not need a carrier to enter the cell); viral carriers such as lentiviruses, adenoviruses and adeno-associated viruses; nonviral carriers such as cationic or neutral liposomes, polyethyleneimines and atelocollagen; and single chain or peptide-targeted nanoparticles [33].

Several authors, using intravenous injections of cholesterol-conjugated single-stranded antagomirs (stabilized by 2'O-methylations) demonstrated that it is possible to inhibit the miRNAs overexpression linked to tumorigenic processes [31]. For example, the inhibition of miR-10b in mice transplanted with a high metastatic breast cancer cell line using this method resulted in a robust inhibition of metastasis to

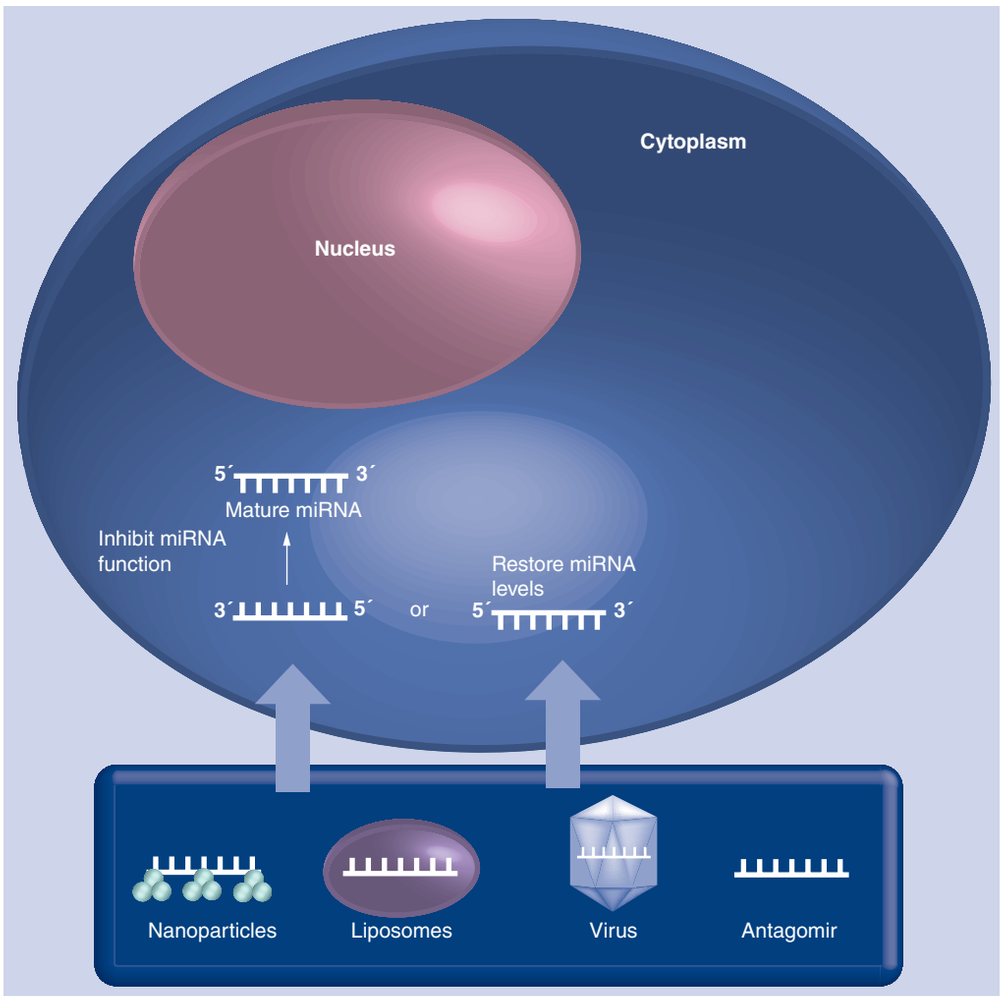

Figure 4. Proposed strategies for miRNA delivery in targeted therapies. Once inside the cell, the miRNAs can serve as antagomirs and inhibit the function of another miRNA, or they can restore the levels of miRNAs that were suppressed or deleted and recover their function.

the lung [67]. The same antineoplasic effects were reported by Park and coworkers. When they inhibited miR-221 using a xenograft model for hepatocellular carcinoma there was a reduction in tumor cell proliferation accompanied by an elevated level of apoptosis [68]. Another way to inhibit oncogenic miRNAs is through the reintroduction of tumor-suppressive miRNAs [33]. This is mainly carried out with the use of lipid-vesicles or by viral delivery of miRNAs. An example of such an application is the reintroduction of miR-26a into a mouse model of hepatocellular carcinoma, where the systemic delivery of miR-26a expressed from an

Executive summary

Epidemiology of renal cell carcinoma

- Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney, accounting for approximately 90% of kidney neoplasms and 3% of all adult malignancies.
- The nonexistence of a standard screening test contributes to the fact that one-third of the patients are diagnosed with local invasive disease or metastatic disease.
- One of the early molecular events of RCC is the loss of *pVHL,* which stops the degradation of HIF and leads to its accumulation in the cytoplasm and further migration to the nucleus where it binds to hypoxia related genes. One of those genes is *TGF-*α.
- TGF-α is involved in the induction of cellular proliferation, migration, invasion and apoptosis by activating the EGF receptor (EGFR) signaling pathway.

miRNAs & cancer

- miRNAs are a family of small ncRNAs that regulate gene expression by sequence-selective targeting of mRNAs, leading to degradation or blockade of mRNA at the post-transcriptional level.
- miRNAs have been identified as key regulators in many biological processes including cell development, differentiation, apoptosis and proliferation.
- Many miRNAs have been identified to act as oncogenes, tumor suppressors or even modulators of cancer stem cells and metastasis formation.

miRNA expression modulation during RCC development

- miR-21 overexpression can increase cell proliferation, migration, invasion and survival. Aberrantly expressed miR-21 regulates the TCF21–KISS1-associated RCC invasion pathway. Cells treated with an antisense miR-21 showed a decreased expression of EGFR, activated AKT, cyclin D and Bcl-2 leading to cell proliferation and differentiation inhibition.
- miR-155 may have different roles during clear cell RCC progression: initially its overexpression is related to an increase in tumor size and later, when the tumor reaches stages III and IV, a suppression of miR-155 occurs and it is related to a poorer prognosis for the patient. This miRNA is induced by hypoxia and plays a role as a component of a network of negative feedback loops that control *HIF-1*α translation.
- miR-210 is overexpressed in serum samples of patients with RCC compared with healthy controls and the average serum level of miR-210 was significantly decreased in patients 1 week after resection surgery, suggesting that this miRNA is released from the tumor tissue into the bloodstream. This miRNA is regulated by HIF-1α, so if we have high levels of HIF-1α we can expect higher levels of miR-210.
- The PI3K/AKT pathway is correlated with apoptosis resistance, increased proliferation, cellular migration and survival signaling. This pathway is activated by the EGFR and leads to the increase of miR-221 and miR-222 expression levels. These miRNAs are associated with radioresistance and epithelial-to-mesenchymal transition. Higher expression levels of miR-221 are also associated with a lower overall survival in patients with RCC.
- miR-1233 has been described as a potential biomarker in RCC, since it was detected an increase of its levels in the serum of RCC patients when compared with healthy controls has been observed. It is proposed that miR-1233 overexpression results in the inhibition or suppression of p53, which in turn will result in HIF-1α activation.

miRNAs as potential biofluid markers for diagnosis & prognosis

- The levels of miRNAs in circulating samples are reproducible and consistent among individuals of the same species, and specific expression patterns of serum miRNAs have already been identified as fingerprints of distinctive human conditions, one of them being cancer.
- miRNAs can be used as potential biomarkers for diagnosis.
- miRNA expression profiles are capable of predicting recurrence rates with accuracy.
- miRNA expression patterns can help distinguish tumor histopathological subtypes.

miRNAs as potential tools for targeted therapy

- There are four types of miRNA delivery strategies to restore miRNA levels in cancer: antagomirs and locked nucleic acids, viral carriers, nonviral carriers and single chain or peptide-targeted nanoparticles.
- Although targeting miRNAs provides an efficient way to modulate gene expression, the efficacy and safety of miRNA-derived drugs must be carefully assessed and will depend on tumor cells type and context.

adeno-associated viral vector resulted in inhibited cancer cell proliferation and induction of apoptosis [69].

Although targeting miRNAs provides an efficient way to modulate gene expression and act as a cancer therapy, the efficacy and safety of miRNA-derived drugs must be carefully assessed and will depend on the tumor cells type and context. One of the biggest problems of using miRNA targeted therapies is related to the fact that a single miRNA can have multiple targets and can influence multiple pathways. By inhibiting a single miRNA we can block an oncogene but we could also suppress a tumor suppressor gene. Furthermore, our current understanding of miRNA effects and function comes mainly from cell culture studies and animal models, and even though this has opened new doors and perspectives in oncology, it is still limited, so it is clear that we must continue to explore the influence of miRNA in the tumor and cellular context and try to implement that knowledge in clinical practice in the near future.

Conclusion

Altered miRNA expressions are useful as biomarkers for diagnosis and prognosis purposes. These findings have opened up a new and interesting field in the screening and monitoring of cancer patients. Despite recent advances in the study of the mechanisms involved in RCC pathophisiology and the identification of the central signaling pathways, other pathways such as the EGFR pathway seem to be implicated in this neoplasm. This pathway can be induced as a consequence of VHL inactivation, and can be modulated by different miRNAs during RCC development. In this review we have highlighted some potential miRNAs that could be involved in the modulation of the EGFR pathway and consequently in RCC development.

Future perspective

In the future, the knowledge of the expression profile of miRNAs could contribute to the development of more effective therapeutic strategies and new targeted therapies that restore normal miRNA networks.

The EGFR signaling pathway seems to have a key role in RCC and further studies are needed to characterize the deregulation of this pathway during RCC development.

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